Carbohydrate content of the acetylcholine receptor from Torpedo marmorata

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CARBOHYDRATE CONTENT OF THE ACETYLCHOLINE RECEPTOR FROM TORPEDO MARMORATA

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SUMMARY

The acetylcholine receptor from Torpedo marmorata electric organs was purified by using standard techniques involving solubilisation of the receptor in the non-ionic detergent Triton X100 followed by affinity purification on an α-toxin affinity column. Specfic activity of receptor preparations were assessed in terms of binding of iodinated α-bungarotoxin which was itself checked to maximise biological activity. Efforts were made to optimise the yield and specific activity of the receptor glycoprotein.

Gas-liquid chromatographic analysis of purified Torpedo AChR revealed the presence of fucose (47.1nmol/mg protein), mannose (122.7nmol/mg protein), galactose (40.3nmol/mg protein) and N-acetyl-D-glucosamine (41.8nmol/mg protein). The presence of glucose was more difficult to confirm as it was not shown to be present in all receptor preparations. The amounts of all monosaccharide residues could be reduced by glycosidase treatment. From alkaline borohydride treatment of the receptor, the oligosaccharides were found to be N-glycosidically linked to the protein.

A lectin-binding assay was developed to detect carbohydrate residues in nitrocellulose blots of SDS gels of Torpedo AChR. Lectin binding to these blots showed that mannose is present in all four subunits while fucose, galactose, (glucose), N-acetylglucosamine and

sialic acid appear to be present in the γ- and δ-chains only.

A further assay was developed to investigate lectin binding to the Torpedo AChR. This assay, the enzyme-linked lectin binding assay (ELBA), was developed from a modification of the ELISA and was successfully used to detect the presence of mannose and fucose in the AChR. Binding of Con A and Lotus tetragonolobus lectins to the Torpedo receptor could be inhibited by mannose and fucose respectively.

A passive haemagglutination test was developed. The AChR was coupled to red blood cells by the chromic chloride method. The bound receptor retained both antigenic activity and the ability to bind αBGT. The AChR-coated RBC were successfully used in a haemagglutination test to determine antibody titre and cross-reactivity of polyclonal and monoclonal anti-acetylcholine receptor antisera. This method of insolubilising the receptor can be useful for ligand binding studies and has a good potential for detecting lectin binding to the receptor and for investigating the possible antigenic nature of the AChR sugar residues.

ABBREVIATIONS

AChR	Acetylcholine receptor
BGT	Bungarotoxin
BSA	Bovine serum albumin
CM	Carboxymethyl
DEAE	Diethylaminoethyl
ELBA	Enzyme-linked lectin-binding assay
ELISA	Enzyme-linked immunosorbant assay
FAB	Fast atom bombardment
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Fuc	Fucose
Gal	Galactose
GalNAc	N-Acetylgalactosamine
GLC	Gas-liquid chromatography
Glu	Glucose
GluNAc	N-Acetylglucosamine
HRBC	Human red blood cells
HRO	Horseradish peroxidase
HPO	Horseradish peroxidase
Man	Mannose
MS	Mass spectrometry
NeuNAc	Neuraminic acid
NMR	Nuclear magnetic resonance
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline

PMSF	Phenylmethylsulphonylfluoride
RBC	Red blood cells
RIA	Radioimmunoassay
SRBC	Sheep red blood cells
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylenediamine
TFA	Trifluoroacetic acid

INTRODUCTION

The successful isolation and characterisation of the nicotinic acetylcholine receptor (AChR) has been facilitated by the availability of a unique tissue - the electric organ of several species of electric fish. The electroplaque cells of electric tissue from Torpedo marmorata are exclusively cholinergic in their innervation and contain densely packed arrays of receptor molecules providing a rich source of material.

The human disease Myasthenia gravis and its animal model, Experimental Autoimmune Myasthenia gravis, both involve an autoimmune response to antigenic determinants associated with the acetylcholine receptor at the post synaptic membrane of the neuromuscular junction. It is accordingly of interest to learn something of the structural basis of the antigenicity exhibited by membrane fragments rich in receptor material.

The Electric Organs of the Electric Fishes (Reviewed by Bennett, 1970)

The electric organs of Torpedo constitute two large, flat, kidney-shaped masses on both sides of the anterior part of the body, their weight ranging from one sixth to one quarter of that of the fish. Embryological studies have shown that the organ derives from striated muscle (specifically, modified brachial muscles) and

receives 6000 nerve fibres from an equal number of neurons clustered on lobes on the dorsal part of the mesencephalon. Although contractile elements are absent from the organs, they have retained their excitability, responding to acetylcholine in a similar way to that of muscle cells.

The electric organs of the electric fishes consist of arrays of large flat cells, the electroplaques, each of which is capable of producing a change in potential on receiving a nerve impulse. The cells are arranged in series and the discharge produced is the summation of the potential generated by each electroplaque (approx. 0.14V/cell). E.electricus and T.marmorata generate potentials of 600V and 40-60V respectively. This ability of the electric rays and electric eels to administer numbing shocks has been known since Roman times, and was the subject of a poem, De Torpedine by Claudian (370-408 AD). A primitive form of electroconvulsive shock therapy was even carried out by placing live Torpedo on the patients head!

The electroplaques of both species consists of a series of highly assymetric giant syncytia each containing several thousand nuclei. Each cell receives nerve terminals on one face only. In Electrophorus, this is the caudal face and in Torpedo, it is the ventral face. The surface areas of both the dorsal and ventral plasma membranes show a considerable increase as a consequence of the proliferation of invaginations and

villosities. In Electrophorus, nerve-endings may establish 10^5 - 10^6 specific contacts. However in Torpedo, innervation is much denser and the innervated membrane less convoluted. There may be as many as 150-300,000 electroplaques in one organ of Electrophorus and thus the total number of synapses in one organ may be in the region of 10^{10} - 10^{11}. All the synapses function with the same neurotransmitter, acetylcholine. The fish receptors show nicotinic pharmacology and so the electric organs from both species (and especially Torpedo) serve as a rich and homogeneous source of this form of receptor. The tissue is also an ideal model for the study of neurotransmission at the neuromuscular junction.

To date, there are but minor differences known between the electroplaque synapse and the neuromuscular junction from higher order vertebrates. At the electron microscope level the synapse of the electroplaque is much simpler than that at the motor end plate of skeletal muscle, with the nerve terminal exposed to a flat subsynaptic membrane without the typical subneural folds associated with the vertebrate postsynaptic membrane (Bourgeois et al., 1978).

The α-Neurotoxins As Probes For The Acetylcholine Receptor

The isolation and characterisation of the acetylcholine receptor was made possible by the use of

α-toxins from the Elapidae (eg cobra and krait) and Hydrophidae (sea snakes) families of snakes. These α-toxins are small compact polypeptides with 60-74 amino acids, possessing a net positive charge. They block neuromusclar transmission without affecting the action potential in nerve or muscle, the release of acetylcholine or its hydrolysis by acetylcholinesterase. The binding of α-toxins can be prevented by d-tubocurarine (a nicotinic antagonist). Two groups of α-toxins have been identified according to their action at the neuromuscular junction. Type I α-toxins (e.g. the α-toxin from Naja naja siamensis) produce a reversible neuromuscular blockade and have been used as ligands for affinity chromatography in the purification of the acetylcholine receptor. Elution of the receptor is effected by agonists such as carbamylcholine (Klett et al., 1973, Lindstrom and Patrick, 1974). Type II α-neurotoxins (e.g. the α-toxin from Bungarus) bind almost irreversibly to acetylcholine receptor and may be radiolabelled to a high specific activity and still retain their pharmocological properties. Radiolabelled α-bungarotoxin (αBGT) is extensively used for detection and quantification of AChR for biochemical analysis. Several procedures have been used for radiolabelling α-neurotoxins (reviewed by Fulpius, 1976). These methods include acetylation with ^{3}H acetic anhydride (Barnard et al., 1971, Chang et al., 1973), radiolabelling with N-succinidyl-(2,3-^{3}H) propionate (Dolly et al., 1981) and

iodination with ^{125}I (Wang and Schmidt, 1980). ^{125}I-αBGT has also been used in autoradiographic studies to examine the density and distribution of the receptor in intact cell membranes. Separation of bound radiolabelled αBGT from free ligand in any assay may be achieved by a variety of techniques: gel filtration on Sephadex G50 (Miledi et al., 1971), ultrafiltration of the receptor-toxin complex in a sucrose density gradient (Lindstrom and Patrick, 1974), differential ammonium sulphate precipitation of the receptor-toxin complex (Meunier et al., 1972) and separation on DEAE-cellulose filter discs (Schmidt and Raftery, 1973, Dolly, 1979). Acetylcholine receptor is normally quantified in terms of moles of αBGT binding sites per gram of protein.

Purification and Characterisation of the Acetylcholine Receptor from the Electric Fishes

Among the first studies on the acetylcholine receptor were those reported by Miledi (1971). As the native receptor interacts with the synaptic membrane hydrophobically, detergent is required to extract it and maintain it in solution. Miledi labelled membrane receptor with radioiodinated αBGT and dissolved the whole complex in Triton X100. It was shown that solubilised receptor protein - ^{125}I-αBGT complex from membranes of Torpedo could be separated from the enzyme acetylcholinesterase by gel filtration (Miledi et al., 1971). This was similarly demonstrated by eluting Electrophorus receptor from an affinity column (Meunier et al., 1971). An hypothesis that the acetylcholine receptor constituted an allosteric site of the enzyme acetylcholinesterase (Changeux, 1966) was thereby disproved (Miledi et al., 1971, and Meunier et al., 1971). Sephadex gel filtration and density gradient centrifugation of the solubilised receptor showed that the protein-detergent complex had a high molecular weight. Conventional protein purification procedures in neutral detergent solution have given significant purification of both the free receptor and toxin-receptor complex. These techniques have been particularly

successful with high specific activity crude extracts from Torpedo electric tissue (Potter, 1973). Improvement of purification and yields resulted from the use of affinity chromatography.

Purification by affinity chromatography has been effected by conjugation of either Naja α-toxin or, synthetic cholinergic ligands, to agarose beads (Olsen et al., 1973, Schmidt and Raftery, 1972, Bisecker, 1973, Karlin and Cowburn, 1973). Originally the α-toxin from Naja nigricollis was used, however this binds the receptor site with too high an affinity and quantitative release of the receptor from the column of α-toxin is more readily achieved by use of the α-neurotoxin from Naja naja siamensis (which has a lower affinity) (Karlsson et al., 1972, Klett et al., 1973, Eldefrawi and Eldefrawi, 1973, Lindstrom and Patrick, 1974). More recently, Lennon et al (1980), have used monoclonal anti-(AChR) IgG coupled to agarose to purify T.californica AChR.

Purification to homogeneity was acheived by additional steps such as sucrose density gradient ultracentrifugation (Lindstrom and Patrick, 1974, Meunier and Changeux, 1973, Meunier et al., 1974), DEAE chromatography (Klett et al., 1973) or electrophoresis (Eldefrawi and Eldefrawi, 1973).

The AChR of the electroplaque has been extensively characterised (for reviews see Karlin, 1980, Changeux, 1981, Conti-Tronconi and Raftery, 1982, Barrantes, 1983,

Popot and Changeux, 1984). The physical properties of the acetylcholine receptor are summarised in Table 1.1. The purified receptor has been shown to be a glycoprotein (see Introduction section on the History of the Glycoprotein Nature of the AChR).

Molecular Weight and Subunit Composition

Determination of the molecular weight of the AChR in solution has proved difficult because of the presence of bound detergent. Bound Triton X100 has been reported as contributing up to 21% (160-170 molecules) of the total mass of the receptor-toxin complex (Meunier et al., 1972) or 0.5g Triton X100 per gram protein (Karlin et al., 1979). Values for the molecular weight have been quoted in the region of 250,000-370,000 (for reviews see Changeux, 1975, Fulpius, 1976, Changeux, 1981, Popot and Changeux, 1984).

Sucrose density centrifugation has shown Torpedo acetylcholine receptor to exist in two forms with sedimentation coefficients of 9S and 13S (Reynolds and Karlin, 1978). The latter form is a dimer of the 9S protein linked by disulphide bonds between the δ-subunits (Sobel et al., 1977, Suarez-Isla and Hucho, 1977). The 13S form has not been detected in Electrophorus (Ruchel et al., 1981).

The number of subunits was the subject of conflicting reports from different laboratories (see

Table 1.1 Physical Properties of the AChR from Fish Electric Organ (Electrophorus and Torpedo) (adapted from Changeux, 1980)

PROPERTY	VALUE	REFERENCES
LIGHT FORM		
SEDIMENTATION COEFFICIENT	9s	Meunier et al 1971, 1972 Raftery et al 1971, 1972
STOKES RADIUS	7nm	Meunier et al 1971, 1972 Raftery et al 1971, 1972 Klett et al 1973
MOLECULAR Wt		
SDS-cross linking	260,000	Bisecker, 1973
SDS-cross linking	230,000	Hucho & Changeux, 1973
Hydrodynamics	250,000	Reynolds & Karlin, 1978
Neutron scattering	240,000	Wise et al., 1979
(Radiation inactivation)	300,000	Lo et al., 1982
RADIUS OF GYRATION		
Neutron scattering	4.61nm	Wise et al., 1979
SHAPE (electron microscope)		
Rosette	9nm	Cartaud et al., 1978 Heuser & Salpeter., 1979
DETERGENT (Triton X100) BINDING		
Hydrodynamics	0.23g/g	Gibson et al., 1976
Osmometry	0.45g/g	Martinez-Carrion et al., 1975
Direct binding	0.37g/g	Wise et al., 1979
Neutron scattering	0.49g/g	Wise et al., 1979

Table 1.1 Cont.

PROPERTY	VALUE	REFERENCES
HEAVY FORM		
HYDRODYNAMIC PARAMETERS	13s, 9nm	Raftery et al., 1972 Potter, 1973
MOLECULAR Wt	500,000	Reynolds & Karlin., 1978
SHAPE (electron microscopy) Doublet of rosettes	2 x 9nm	Cartaud et al., 1980 Wise et al., 1981
SUBUNIT MW:- 40, 50, 60, 65K		
ISOELECTRIC POINT	4.9	Raftery et al., 1972
CARBOHYDRATES:- Glucosamine, mannose, glucose, galactose, sialic acid, 75 residues per molecule		Vandlen et al., 1979
PHOSPHORUS In vitro phosphorylation		Teichberg et al., 1977 Gordon et al., 1977 Saitoh & Changeux, 1980
Chemical analysis, O-phosphoserine		Reynolds & Karlin, 1978 Vandlen et al., 1979

Conti-Tronconi and Raftery, 1982). However the use of potent protease inhibitors appears subsequently to have condemned some early reports as misinterpretations resulting from proteolysis (Karlin et al., 1976, Raftery et al., 1976, Lindstrom et al., 1978). The 9S form of the AChR has now been established to consist of five subunits α_2, β, γ, δ having molecular weights, as assessed by SDS-PAGE, of 40,000, 50,000, 60,000, and 65,000 respectively (Reynolds and Karlin, 1978, Lindstom et al., 1979, Raftery et al., 1980). Recently the α, β, γ and δ subunits have been produced from cloned DNA and the amino acid sequences of the chains have been elucidated (Noda et al, 1982, 1983a, 1983b). Considerable homology has been found between subunits.

Additional protein components of molecular weight 43,000 and 90-150,000 have been consistently associated with the AChR; the higher molecular weight component is considered to be a component of the Na^+, K^+-ATPase. A 95K component has also been shown to be derived from contaminants (Lindstrom et al., 1979). Early evidence suggested that the 43K protein may be involved in the binding of non-competitive blockers (Sobel et al., 1978, Blanchard and Raftery, 1979, Neubig et al., 1979) but recent reports (Barrantes et al., 1980, Lo et al., 1980, Wennogle and Changeux, 1980, Gordon et al., 1983) indicate that the 43K protein is a peripheral protein associated with the cytoplasmic face of the membrane, possibly having a structural role. More recently

Barrantes (1983) has summarised the information concerning 43K protein as follows. It

1) appears to be established in close contact with the acetylcholine receptor protein presumably in a region covering part of the α_1, γ and δ subunits

2) participates in the thiol-dependent receptor aggregational states

3) has a possible influence on the freedom of motion of the acetylcholine receptor protein, both in terms of its rotational and translational mobility, and

4) possibly has an influence on the susceptibility of the acetylcholine receptor to thermal denaturation (Saitoh et al., 1979) or enzymic attack (Klymkowsky et al., 1980, Wennogle and Changeux, 1980).

Functions of the subunits of the acetylcholine receptor

Specific binding with the affinity ligands 4-(N-maleimido)-benzyl trimethylammonium iodide (MBTA) (Reiter et al.,1972) and bromoacetylcholine (Silman and Karlin, 1969) to the acetylcholine site, after reduction of disulphide bonds by dithiothreitol, labels the 40,000 subunit, which is accordingly considered to bear all or part of the physiologically significant acetylcholine binding site. It appears, however, that only 50% of α-toxin binding can be inhibited with MBTA, suggesting that only one of the α-subunits has been labelled (Reed et al., 1975, Karlin et al., 1976). Bromo ^3H

acetylcholine on the other hand, labels all the α-toxin sites, although binding to membrane fragments of Torpedo is biphasic (Wolosin et al., 1980). To explain these results various theories have been proposed, including changes in ligand affinity with occupancy, heterogeneity in the acetylcholine binding subunits of reduced receptor and the ability of the -SH to react in different states of the receptor.

The function of the β, γ and δ subunits of the acetylcholine receptor is still not clear. Cloned mDNAs encoding the four subunits of T.californica acetylcholine receptor have been inserted into Xenopus oocytes from which it was concluded that all four chains are required to elicit a normal nicotinic response to acetylcholine, whereas only the α-subunit is indispensable for α-bungarotoxin binding activity (Mishina et al., 1984).

Phosphorylation of the Acetylcholine Receptor

Phosphorylation of Torpedo and Electrophorus AChR has been reported in a membrane fraction and in crude detergent extracts (Teichberg and Changeux, 1976, Gordon et al., 1977, Saitoh and Changeux, 1980). The degree of phosphorylation has been shown to increase during development (Saitoh and Changeux, 1981). Purified AChR from Torpedo contains about 10 phosphate groups, possibly attributable to the phosphoserine present in each chain (Vandlen et al., 1979).

The structure of the acetylcholine receptor within the membrane

The location of subunits of the nicotinic acetylcholine receptor in the phospholipid bilayer of the membrane has been investigated by using a number of techniques. X-ray diffraction analysis (Ross et al., 1977, Potter and Smith, 1977) has been used in an attempt to characterise the three-dimensional structural organisation of the AChR. Electron microscopy of negatively stained preparations, in which the receptor images were identified immunologically, indicate that the receptor extends approximately 5.5nm from the extracellular surface of the membrane with a 1.5nm exposure on the cytoplasmic face (Klymkowsky and Stroud, 1979, Schiebler and Hucho, 1978, Kistler et al., 1982).

Circular dichroism studies suggest that 34% of acetylcholine receptor is α-helical (Moore et al., 1974). A combination of electron microscopy and single particle image averaging has indicated that the two α subunits are diametrically apposed with about 50A separating them (Zingsheim et al., 1982). Conformation by cross-linking studies appears to show that all four chains (but predominantly the α and δ) are in close proximity (Karlin, 1980, Hucho, 1981). The formation of diamide induces trimers of the AChR in receptor-rich membranes. Disulphide bonds link alternating pairs of δ chains and β chains. An angle of 50-80^{o} between the β-β disulphide bond and the δ-δ bond has been revealed by scanning electron microscopy (Wise et al., 1981). More recently, Karlin et al. (1983) have elucidated the arrangment of the five subunits. Using Naja naja siamensis α-toxin labelled with biotin and avidin they deduced that the most likely orientation of the subunits is in the order α, γ, α, δ, β. Various studies on the orientation of specific subunits with respect to the membrane, including lactoperoxidase-catalysed iodination (Hartig and Raftery, 1977, Cohen et al., 1980), peptide cross-linking (Witzemann and Raftery, 1978, Karlin et al., 1979, Karlin, 1980), proteolytic degradation (Strader and Raftery, 1980, Wennogle and Changeux, 1980, Anderson and Blobel, 1981, Wennogle et al., 1981) and electron microscopy (Brisson and Unwin, 1985) have indicated that all chains are exposed extracellularly whilst the 43K

protein is located on the cytoplasmic face. The subunit organisation is shown in Figure 1.

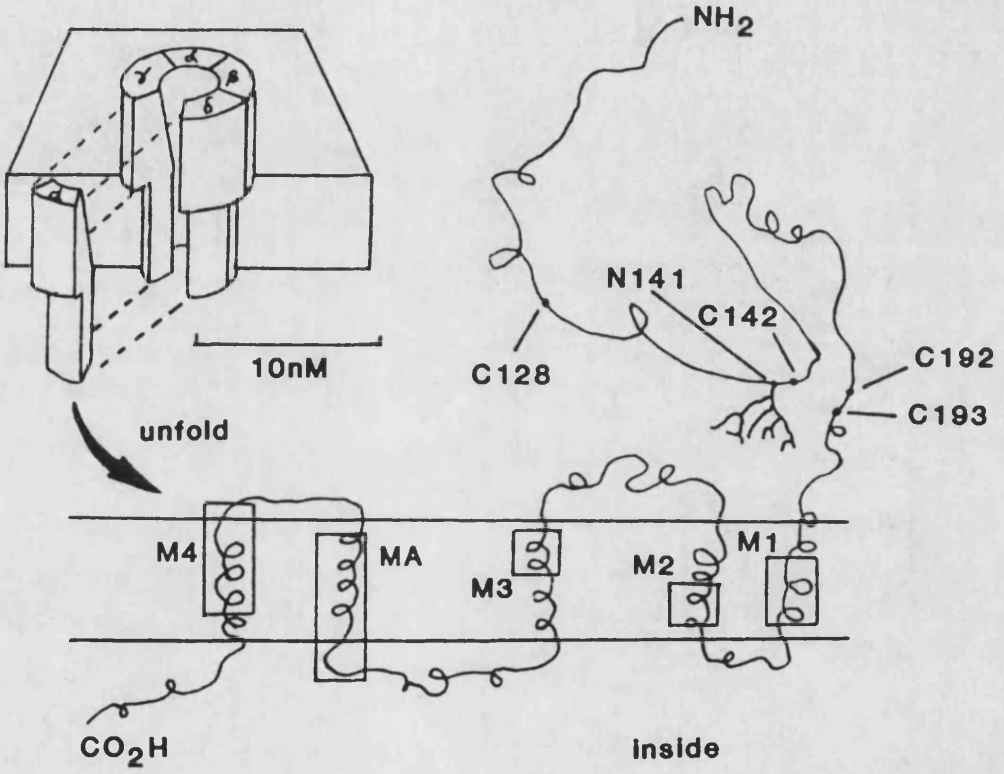
Binding of monoclonal antibodies (raised against acetylcholine receptor from electric fish) to skeletal muscle of Rana and Xenopus, followed by electron microscopy, has confirmed that parts of all four subunits are exposed on the cytoplasmic surface (Sargent et al., 1984). Results of initial studies to determine the extent of exposure were controversial due to the perturbative methods employed in such investigations.

As sequence information on the acetylcholine receptor channel has become available, a number of laboratories have proposed structures for the membrane-spanning portions of the channel. There is now evidence to confirm a model of the receptor as being a charge-lined pore with alternating regions where positive and negative charges predominate (Young et al., 1985, Ratnam and Lindstrom, 1984, Criado et al., 1985).

History of the Glycoprotein Nature of the Acetylcholine Receptor

Eldefrawi and Eldefrawi (1973) assayed the carbohydrate content of purified AChR from T.marmorata by the Anthrone colourimetric method. Equal amounts of carbohydrates were found in the AChR preparations and in blanks of Ringer and in BSA processed through the affinity adsorption procedure. Eldefrawi and Eldefrawi

Figure 1. (Top left) Cut away view of AChR in the cell membrane. The disposition of subunits around the ion-channel is arbitrary and not conclusively established. (Bottom and right) Standard methods of empirical protein secondary structure prediction and hydrophilicity analysis have led to models for the orientation of an individual subunit in the membrane in which hydrophobic membrane-spanning stretches of the polypeptide are bounded on both sides by stretches of hydrophilic amino acids, one of which lies on the extracellular face and the other on the cytoplasmic face of the postsynaptic membrane. One model proposes four hydrophobic (M1-M4) and one amphipathic (MA) transmembrane helices as shown: an amphipathic helix has hydrophobic groups along one face and hydrophilic groups along the other face. The latter have been postulated to contribute to the lining of the ion channel. The alpha subunit (shown here) has a binding site for ACh close to a cysteine (C192) that participates in a disulphide bond with one of the other three cysteines (C128, C142 and C193) in the extracellular portion of the protein. An N-glycosylation site (N141) is important for the assembly, and possibly the function, of the receptor. (Finer-Moore and Stroud, 1984).


γ
α
β
δ
10nM
unfold
NH2
N141
C142
C128
C192
C193
M4
MA
M3
M2
M1
CO2H
Inside

thus concluded that the only carbohydrates in the receptor preparation came from the Sepharose used in affinity adsorption. However, in the same year, Raftery et al (1973) proved that the purified AChR from T.californica was a glycoprotein (by carbohydrate staining of SDS gels), and that it contained approximately 7% total neutral sugars. Mannose, galactose and N-acetyl-D-glucosamine were tentatively identified. Tests for sialic acid proved negative. Staining for protein with Coomassie Blue and for carbohydrate with periodic acid Schiffs reagent demonstrated coincidence of the protein and sugar stains. Two major and two minor polypeptide chains were detected with molecular weights in the neighbourhood of 40,000, the higher molecular weight polypeptides contained more sugar-staining material than the lower molecular weight ones.

Meunier et al (1974) investigated the presence of carbohydrates by studying the interaction of purified AChR from Electrophorus electricus with a variety of plant lectins, proteins known to bind sugars. Reaction of the receptor with Concanavalin A, a lectin which combines specifically with mannosyl residues, was assessed by first precipitating the AChR with Con A followed by centrifugation. The supernatants were then assayed for receptor activity by the Millipore filtration assay (Schmidt and Raftery, 1973). Interaction of receptor preparations with other lectins (from Triticum vulgaris, Ricinus culinaris, Phaseolus vulgaris and Lens

culinaris) was achieved by mixing purified AChR with the lectins immobilised on Biogel P-300. After incubation and centrifugation, the supernatants were assayed by the Millipore assay. Meunier et al found that Con A precipitated the nAChR and, in addition, inhibited the binding of ^{3}H-labelled toxin to receptor. As there was incomplete inhibition of ^{3}H-labelled toxin binding to the receptor protein by Con A they suggested that this might indicate an heterogeneity of the carbohydrate moiety of the receptor molecule. Experiments with the immobilised lectins showed binding of the receptor to Phaseolus vulgaris and Lens culinaris (specific for N-acetyl-D-galactosamine and D-mannose respectively). They detected no binding to Ricinus communis or Triticum vulgaris (specific for D-galactose and di-N-acetyl-chitobiose respectively). These preliminary investigations suggested that the AChR from E.electricus contained at least D-mannose and N-acetyl-α-D-galactosamine.

Later the same year Moore et al (1974) found (using an amino acid analyser) that the acetylcholine receptor from T.nobiliana contained glucosamine (about 3.8% w/w protein), while Michaelson et al (1974) found that from T.californica to contain N-acetyl-D-glucosamine (using an amino acid analyser) and approximately 5% neutral sugars (as measured by the phenol sulphuric acid method). Analysis of the neutral sugars by ion-exchange procedures revealed the presence of mannose, galactose and glucose

in the relative proportions of 10:2:1. They also reported that ^{125}I-αBGT-AChR complex formation could be inhibited by the lectins from Ricinus communis and Canavalia ensiformis suggesting the presence of D-mannose and D-galactose in the receptor molecule.

Gas-liquid chromatography was performed on the intact receptor from T.marmorata by Mattsson and Heilbronn (1975). This examination revealed a total carbohydrate content of 38 ± 12μg/mg nAChR. The carbohydrates consisted of mannose (50-70% of the total sugar), galactose (10-20%) and glucose (10-30%). N-Acetylglucosamine was also shown to be present. Karlin et al (1975) confirmed the presence of sugars on the then known three subunits of Electrophorus and on the four subunits of Torpedo californica by periodic acid Schiffs staining of polyacrylamide gels.

A single set of gas-liquid chromatography analyses showed all four subunits of T.californica to be glycosylated (Lindstrom et al, 1979). They showed all the subunits to contain mannose, galactose, glucose and glucosamine. Sialic acid was present in the γ- and δ-subunits but it was suggested that the traces of sialic acid observed in the α- and β-subunits were probably insignificant.

Vandlen et al (1979) performed a slightly more exhaustive study of the sugars in the receptor from T.californica. Quantitative analysis of neutral sugars was achieved by ion-exchange chromatography (Heller and

Raftery, 1975) or by the phenol sulphuric acid colorimetric reaction for carbohydrates. Sialic acid was determined by the resorcinol and thiobarbituric acid colorimetric methods. Amino sugars were determined during amino acid analysis. As had been shown before (Karlin et al, 1975), all four subunits stained positively for carbohydrate with the PAS stain. The neutral sugars, mannose, galactose and glucose, were found to be present in a molar ratio of 10:1:2 (total weight, by ion-exchange chromatography, of 2.7 ± 0.3%). Assayed by the phenol sulphuric acid method, a value of 194 nmol of carbohydrate per mg, or 3.5% by weight was obtained. The receptor was also found to contain 0.9 mol percent sialic acid, which was detectable in all subunits.

Fragments of the subunits of the AChR from T.marmorata were studied for their ability to bind Con A and Phaseolus vulgaris lectin after selective proteolysis of the intact receptor (Wennogle and Changeux, 1980). These investigations revealed that all subunits and fragments derived from them bound Con A. The 43,000 peripheral protein was not labelled. Results obtained with Phaseolus vulgaris lectin showed that only the 50,000 and 66,000 chains bound this lectin. These results were obtained by autoradiography of polyacrylamide gels of treated and untreated AChR membranes labelled with iodinated lectins.

Purified nAChR from the electric organ Narke

japonica has been found to contain as much as 18% (by weight) carbohydrate (Ishikawa et al, 1980).

Further evidence for the presence of sialic acid on the nicotinic acetylcholine receptor of T.marmorata was provided by Bersinger et al (1983a). Quantitative analyses of the sialic acid liberated by extensive neuraminidase treatment of nAChR were performed on receptor from fresh and frozen Torpedo electric organs. For these two sources of receptor, they gave, respectively, 1.1 ± 0.4 and 0.7 ± 0.1 moles sialic acid per mole nAChR, calculated from the specific αBGT binding capacity. Binding of iodinated AChR to various lectins (Con A, Lens culinaris, Triticum vulgaris, Helix pomatia, Ricinus communis) revealed the presence of mannose, N-acetyl-glucosamine, N-acetyl-galactosamine and galactose.

Possible sites of glycosylation have come from work done by Noda et al (1982, 1983a, b) following cloning and sequencing of cDNA for the subunits of T.californica. The only possible site of N-glycosidic linkage in the α-subunit is the asparagine residue in the sequence Asn - Cys - Thr at positions 141 - 143 (Noda et al, 1982). This sequence is contained within region b, a site at which amino acid sequences are identical in the α-, β- and γ-subunits, and which is exposed on the extracellular surface of the receptor molecule (Noda et al, 1983a). Further work by the Numa group (1983b) has revealed that there is a single possible N-glycosidic

linkage site (position 141) in the β-subunit, 3 possible glycosylation sites (positions 70, 141 and 306) in the δ-subunit and 4 possible glycosylation sites (positions 70, 141, 306 and 354) γ-subunit. Positions 306 and 354 in the γ-subunit are assigned to the cytoplasmic side of the membrane (Noda et al, 1983b).

Both junctional and extrajunctional nAChR from rat diaphragm muscle were found to bind Con A (Brockes and Hall, 1975) showing that, like the receptor from the electric fishes, mammalian acetylcholine receptor is a glycoprotein. Further confirmation of the glycoprotein nature of mammalian AChR was found by Shorr et al (1978), who found that, out of the four subunits (41,000, 50,000, 60,000 and 100,000 MW) from cat skeletal muscle AChR, only the 41,000 subunit exhibited PAS staining. However ^{125}I-labeled Con A has been shown to bind to 3 subunits (42, 49 and 55K subunits) of the foetal calf receptors (Conti-Tronconi et al, 1982). Also, Gotti et al (1982) found a large amount of amino sugars (2 mol% N-acetylglucosamine, 8 mol% N-acetylgalactosamine) to be consitently present in amino acid analysis of 4 preparations of foetal calf muscle. Separation of Electrophorus muscle AChR by SDS PAGE and subsequent treatment of the gels with PAS stain and protein blots with iodinated Con A has revealed that all four subunits are glycosylated (Gullick and Lindstrom, 1983). Also, treatment of the receptor with neuraminadase resulted in an increase in the mobility of all the subunits,

suggesting the presence of sialic acid residues. It has also been shown (Hall et al, 1983) that the adult and foetal forms of muscle AChR can be distinguished by a myasthenia gravis autoantibody against carbohydrate. Turnbull et al (1985) found, through binding of ^{125}I-Con A, that the 4 subunits of human skeletal muscle AChR were glycosylated. They also noted a marked difference in the degree of glycosylation between the foetal and adult receptor. The difference in gylcosylation was noted when the electrophoreses patterns of foetal and adult receptors were incubated with ^{125}I-labelled Con A; although, in both cases, all four major subunits showed bound radioactivity, the 44,000 band of foetal receptor was much less strongly labelled than that of the adult form.

Evidence above indicates that muscle AChR shows a similar degree of glycosylation as that of electric organ receptor. Analysis of calf cDNA coding for the subunit precursors of muscle receptor has revealed remarkable homology in the sites of N-glycosylation in muscle and electric organ receptor. Cloning and sequence analyses of calf AChR cDNA encoding the α- and β-subunits have shown the asparagine residue (residue 141) to be the only possible site of N-glycosidic linkage (Noda et al, 1983c, Tanabe et al, 1984). In addition to asparagine residue 141, the γ-subunit has 3 other possible sites (residues 30, 306 and 354), two of which (residues 141 and 306) are conserved in its Torpedo electric organ counterpart:

only residues 30 and 141 are assigned to the extracellular side of the membrane (Takai et al, 1984). The δ-subunit has 2 possible sites of N-glycosylation (residues 76 and 141) (Kudo et al, 1985).

Function of the Carbohydrate Component of the Acetylcholine Receptor

Although over the years it has becoming increasingly evident that the nicotinic acetylcholine receptor from various sources was a glycoprotein, and in several instances the nature of this carbohydrate component has been elucidated, its function was still unclear. Wonnacott *et al* (1980a,b) investigated the interrelationship of Con A binding and the antigenic and α-toxin binding sites on the acetylcholine receptor from *T.marmorata*. They reported that, despite treatment to remove carbohydrates from the receptor (periodate oxidation and incubation with *T.foetus* glycosidases, β-D-galactosidase and α-L-fucosidase), neither loss of antigenicity nor loss of toxin-binding could be attributed to the effects of these agents. They concluded that the glycosylated portion of the acetylcholine receptor may serve some general function such as that of maintaining the orientation of the receptor in its membrane environment.

Competition studies with lectins and selected monoclonal antibodies (Bersinger *et al*, 1983b) indicated that carbohydrate is located distant from the cholinergic binding site on the Torpedo acetylcholine receptor. Nevertheless, a lectin specific for N-acetylglucosamine was capable of partially inhibiting the binding of a monoclonal antibody specific for the toxin site of nAChR.

An inhibitory effect of Con A on the binding of a monoclonal antibody directed at the major immunogenic region of the AChR was also observed, indicating the presence of mannose-rich carbohydrates close to the major immunogenic region of the receptor.

Evidence to support the theory that carbohydrate stabilises the acetylcholine receptor in the membrane came from work done by Prives and Olden (1980). The function of carbohydrate components of glycoproteins can be conveniently studied in cultured cells by the use of tunicamycin, an antibiotic which specifically inhibits protein N-glycosylation. Prives and Olden found that the treatment of muscle cells with tunicamycin resulted in three marked effects: firstly the glycosylation of protein was almost abolished, secondly accumulation of AChR in intact muscle cells was inhibited, lastly, acetylcholine receptor incorporated into surface membranes of tunicamycin-treated muscle cells displayed a markedly enhanced rate of degradation. In contrast they found that there was no evidence that tunicamycin treatment of cells affected acetylcholine receptor biosynthesis, intracellular transport or incorporation into surface membranes. Leupeptin, an inhibitor of intracellular proteases, markedly increased accumulation of acetylcholine receptor on the external surface of muscle cells treated with tunicamycin. The conclusions from these experiments were that impairment of protein glycosylation prevented accumulation of acetylcholine

receptor by increasing its susceptibility to degredation by cellular proteases.

The absence of oligosaccharide side chains (as a result of tunicamycin treatment) was found to have a dramatic effect on the level of toxin-binding sites in muscle cell surface membranes (Merlie et al., 1981a). Two reasons have been suggested for this: increased degradation of assembled receptor molecules (first proposed by Prives and Olden, 1980, see above) and failure of the non-glycosylated subunits to assemble into a functional toxin-binding molecule. The latter theory (from Merlie et al., 1981a) contradicts the evidence of Prives and Olden, 1980).

Blocking N-linked glycosylation seems to have a wide range of effects depending on the specific protein, a primary effect being at the level of protein folding or tertiary structure. Gibson et al. (1979) have shown that, in the case of strains of vesicular stomatis virus the assembly of which is particularly sensitive to tunicamycin, the newly synthesised G protein aggregates and is insoluble in Triton X100 extracts. Merlie et al (1981b) had shown that the subunit of the acetylcholine receptor is synthesised on membrane polysomes of the rough endoplasmic reticulum and that glycosylation occurs co-translationally at a site close to the NH_2 terminus. In vivo, the glycosylated subunit transits a precursor pool before acquiring αBGT binding activity 15-30min after completion of synthesis (Merlie and Sebbane, 1981).

When glycosylation is inhibited, the subunit does not transit this pool and become assembled. This may be analogous to the synthesis and processing of vesicular stomatitis virus G protein. When cells are treated with tunicamycin the virus G protein is not transferred to the Golgi apparatus. On the basis of similarities of the assembly of acetylcholine receptor with that of other glycoproteins, Merlie and Sebbane (1981) concluded that the receptor subunits are normally assembled in the Golgi apparatus (Fambrough and Devreotes, 1978). Furthermore, the reason for the apparent inefficiency of assembly of acetylcholine receptor subunits in normal cells treated with tunicamycin may be the inefficiency of transport of subunits to the Golgi (Merlie et al, 1981a). Merlie et al (1981a) reasoned that if (in tunicamycin-treated cells) assembly is indeed blocked because the structurally abnormal nonglycosylated α-subunit is not being transported to the site of assembly, the small amount of assembly which is rapidly terminated may reflect an α-subunit which is quantitatively different in its potential to be transported. For example, these α-polypeptides may have been synthesised at sites which are closer to the site of assembly. An alternative explanation of the effect of tunicamycin on acetylcholine receptor assembly is that it preferentially inhibits the synthesis of the β-, δ- and/or γ-subunits and it is the lack of one or more of these which is the limiting factor in assembly.

Further evidence showing that glycosylation is required for production of functional receptor comes from studies using Xenopus oocytes. Sumikawa et al (1981) showed that acetylcholine receptor could be assembled by Xenopus oocytes injected with heterogenous mRNAs for Torpedo receptor in contrast to the situation in cell-free systems (Mendez et al., 1980). The receptor was found to be intact, be multimeric and show properties characteristic of native AChR, including binding of αBGT. The acetylcholine receptor produced by Xenopus oocytes was glycosylated (it bound Con A-Sepharose), although, the γ-subunit may not be correctly glycosylated and/or processed correctly as it was found to be slightly larger than that in the purified native acetylcholine receptor. Tunicamycin blocked active receptor production (Sumikawa et al., 1983).

Briefly, from the evidence to date there appear to be a number of possible functions for the carbohydrate portion of the acetylcholine receptor. Firstly, it may be implicated in the antigenicity of the receptor in Myasthenia Gravis (Bersinger et al, 1983) although this is thought to be unlikely (Wonnacott et al, 1980a). Secondly, it may be involved in stabilising the receptor subunits in the membrane (Prives and Olden, 1980). Thirdly, it has been suggested that glycosylation of the receptor is essential for its assembly and insertion into the membrane (Merlie et al, 1981a).

Glycoproteins and their Characterisation

The presence of oligosaccharide chains covalently attached to the peptide backbone is the feature which distinguishes glycoproteins and accounts for some of their characteristic physical and chemical properties. Glycoproteins occur in fungi, green plants, viruses, bacteria and in higher animal cells where they form a variety of functions. They make up a large and heterogeneous group. They occur as soluble secreted molecules such as plasma glycoproteins, protein hormones, immunoglobulins, mucins, blood-group substances and also in collagens and basement membranes. Current interest in membrane structure has drawn the attention of many researchers to the fact that glycoproteins also occur in an insoluble form as components of cell membranes. The fluid mosaic model of membrane structure (Singer and Nicolson, 1972) emphasised the occurrence of integral proteins, the structure of many of which have recently been studied and shown to include carbohydrate.

The function of the glycans of the glycoproteins are unclear although various theories have been postulated, these include:

1) an involvement in polypeptide processing
2) intracellular transport of glycoproteins
3) catabolism and clearance of glycoproteins
4) cell adhesion
5) antigenicity

Many polypeptides or proteins are synthesised as giant precursors that are subsequently cleaved by specific proteolysis into mature, biologically active products. The glycans of glycosylated polypeptides are bulky substituents that could possibly play important roles in controlling fragmentation of these precursors. This does appear to happen in the pituitary gland where cells, treated with tunicamycin to block glycosylation, produce very little of the biologically active peptides.

One of the earliest functions ascribed to protein glycosylation was to facilitate secretion. Later refinements of this simple hypothesis propose that glycosylation is principally involved in the insertion of proteins into membranes and their transport by membrane flow to other intracellular membranes and to the surface. A commonly observed feature of tunicamycin-treated cells is a highly distended endoplasmic reticulum engorged with unglycosylated proteins that would normally be swept into other cellular sites.

Proteins within cells are degraded in a controlled manner during normal growth and differentiation (Dean, 1978). The degradation and turnover of proteins is determined in part by inherent features, (e.g. conformation of the polypeptide) but there is considerable evidence that the carbohydrate moieties also regulate the catabolism of glycoproteins. For instance in the liver, plasma membrane glycoproteins turn over more rapidly than non-glycosylated proteins and in

cultured cell lines the plasma membrane glycoproteins are replaced almost entirely under non-growing conditions within a few hours (Hughes, 1976).

Cell adhesion is a property of fundamental importance in the formation of tissues and organs during embryogenesis. Processes involved in embryonic development depend on specific intercellular recognition and selective adhesion between cells. Cells dissociated from various organs and mixed in suspension tend to reform aggregates of cells in a tissue-specific manner. Interference with normal glycosylation often prevents development, (e.g. in sea urchin embryos as well as in some developing mammalian systems such as kidney). Hence glycoproteins are assumed to play important roles in many of these adhesive interactions leading to normal development. If carbohydrate moieties of glycoproteins are involved in cell adhesion, two types of complementary interactions are possible. The first is between carbohydrate chains by a process analogous to the chain-chain interactions of many linear polysaccharides (Rees, 1977) and the second may be between a carbohydrate moiety and a carbohydrate-binding protein, e.g. a lectin.

Carbohydrates have long been suspected of being involved in antibody/antigen interactions. Antibodies have been produced by hybridomas to surface antigens of embryonic cells. The antigens, however, seemed elusive and could not be readily identified by the usual techniques including immune precipitation and

polyacrylamide gel electrophoresis. Several of them, including some oncofetal (tumour-associated) antigens, have been shown subsequently to be carbohydrate sequences on high relative molecular mass glycoproteins (or polysaccharides). The antigens responsible for an autoimmune haemolytic disorder (Feizi and Hadler, 1983) are now known to be carbohydrate structures on glycoproteins and glycolipids of erythrocytes and other cells types (Feizi, 1981, Uemura et al., 1983) consisting of repeating units of N-acetyllactosamine (Gal-β(1→4)-GlcNAc). Various antigens associated with cells of a non-cancerous (e.g. erythrocytes, gastric mucosa, human seminal fluid proteins etc) and of a cancerous nature have been identified by monoclonal antibodies and shown to comprise carbohydrate structures.

Analysis of Oligosaccharides

In view of the possible involvement of carbohydrate in some of the biological functions displayed by glycoproteins, a great deal of effort has been extended in recent years to determine the structure of the oligosaccharide chains of both soluble and membrane glycoproteins. The different monosaccharides present in the carbohydrate portion of membrane glycoproteins are few and include D-galactose, D-mannose, D-glucose (rarely), L-fucose, N-acetyl-glucosamine, N-acetyl-D-

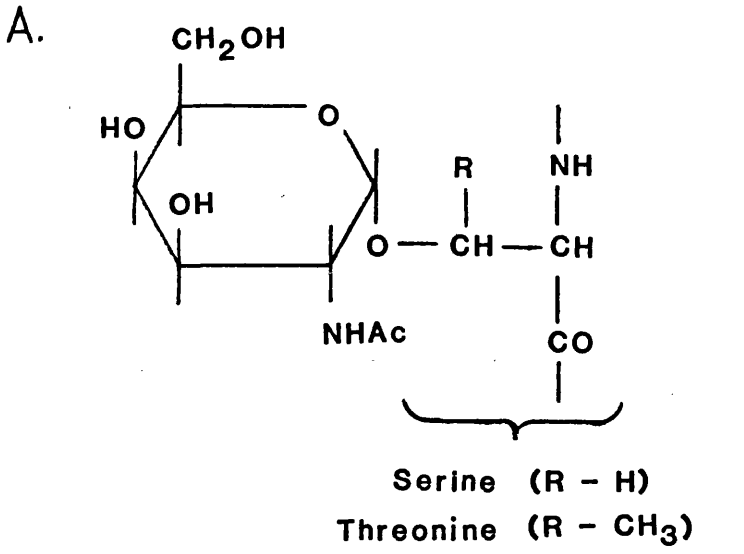
galactosamine and sialic acid, arranged in oligo-saccharide side chains of varying sizes and glycosidically-linked to a polypeptide backbone.

Membrane glycoproteins have been investigated by extraction of the membranes with dissociating reagents (Juliano, 1978) followed by purification. Other glycoproteins have been studied by more indirect methods such as the use of lectins (Lotan and Pertlow, 1979) and cytochemical reagents (Juliano, 1979) acting on the intact membrane.

One of the chemical techniques employed to identify a glycoprotein involves the periodic acid-Schiff (PAS) reagent. The PAS stain for carbohydrate depends on periodate oxidation of carbon-carbon bonds bearing vicinal hydroxyl groups to give carbonyl groups. These will react with Schiffs reagent (leucofuchsin) yielding a pink or purple Schiffs base.

Only a few side chains of the amino acid residues usually found in proteins are suitable for the attachment of mono- or oligo-saccharides. A very common linkage involves the C(1) of N-acetylglucosamine and the amide group of asparagine. The asparagine is part of an extended polypeptide sequence and the N-acetylglucosamine residue forms the point of attachment of other monosaccharides. The carbohydrate chains attached to polypeptides in this fashion are N-glycans (Figure 2B).

The second class of linkage sugars are O-glycans joined through C(1) by glycosidic bonds to the



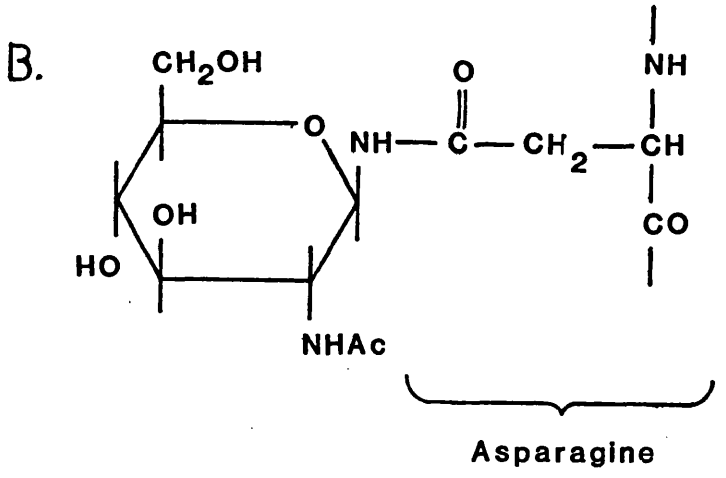


Figure 2. Carbohydrate linkages. A. O-glycosidic linkage to serine or threonine. B. N-glycosidic linkage to asparagine.

hydroxylated side chains of serine, threonine, hydroxylysine or hydroxyproline (Figure 2β). The latter two amino acids are commonly found in plant glycoproteins. Mannose linked to serine and threonine forms the point of attachment of carbohydrate chains in glycoproteins synthesised by yeasts and moulds and has recently been detected in mammalian glycoproteins as a minor components (Finne et al., 1979). In glycoproteins of higher organisms however, the sugar most frequently found linked to serine and threonine is N-acetylgalactosamine.

The structures of some glycopeptides which contain O-glycosidic linkages involving N-acetylgalactosamine as the linkage sugar are shown in Table 1.2. N-Glycosidically-linked (to asparagine) oligosaccharide units are usually found to be of two types. The first contains only mannose and N-acetylglucosamine and is called high-mannose or simple type (Figure 3). The second (complex type) contains a variable number of outer chains linked to a β-mannosyl-di-N-acetylchitobiose unit, the same structure that occurs in the inner region of typical high-mannose oligosaccharides (Figure 3).

Detailed information about the isolation and structural characterisation of membrane glycoproteins and glycopeptides can be found in a number of recent reviews (Sturgess et al., 1978, Tanner, 1978, Kornfeld and Kornfeld, 1980).

Glycoproteins can be separated and analysed for

Table 1.2 Glycopeptides linked through N-acetyl-galactosamine to hydroxyl groups of serine and threonine. (After Kornfeld and Kornfeld, 1980)

Structure	Glycoprotein
A NANA(α2.6)→GalNAc→Ser(Thr) (NGNA)	Submaxillary mucins
B Gal(β1.3)←→GalNAc→Ser(Thr)	"Antifreeze" glycoprotein of antarctic fish: human IgA: subunit HCG: cartilage keratin sulphate: epiglycanin of TA_3.HA cells: lymphocyte, RBC and milk fat globule membranes
C Gal(β1.3)→GalNAc→Ser(Thr) ↑(α2.3) NANA	Bovine kininogen: epiglycanin of TA_3.HA cells: B_{16} melanoma cells
D Gal(β1.3)→GalNAc→Ser(Thr) ↑(α2.3) NANA ↑(α2.6) NANA	Fetuin: human RBC membrane sialoglycoprotein, bovine kininogen: rat brain: milk fat globule membrane: human platelet membrane
E Gal→GlcNAc→Gal→ GalNAc→Ser(Thr) ↑(α2.3) NANA	Epiglycanin
F Gal(β1.3)→GlcNAc(β1.3)→GalNAc ↑(1.6) Ser(Thr) GlcNAc ↑(1.4) Gal	Human gastric mucin: core region of human and hog blood group substance.

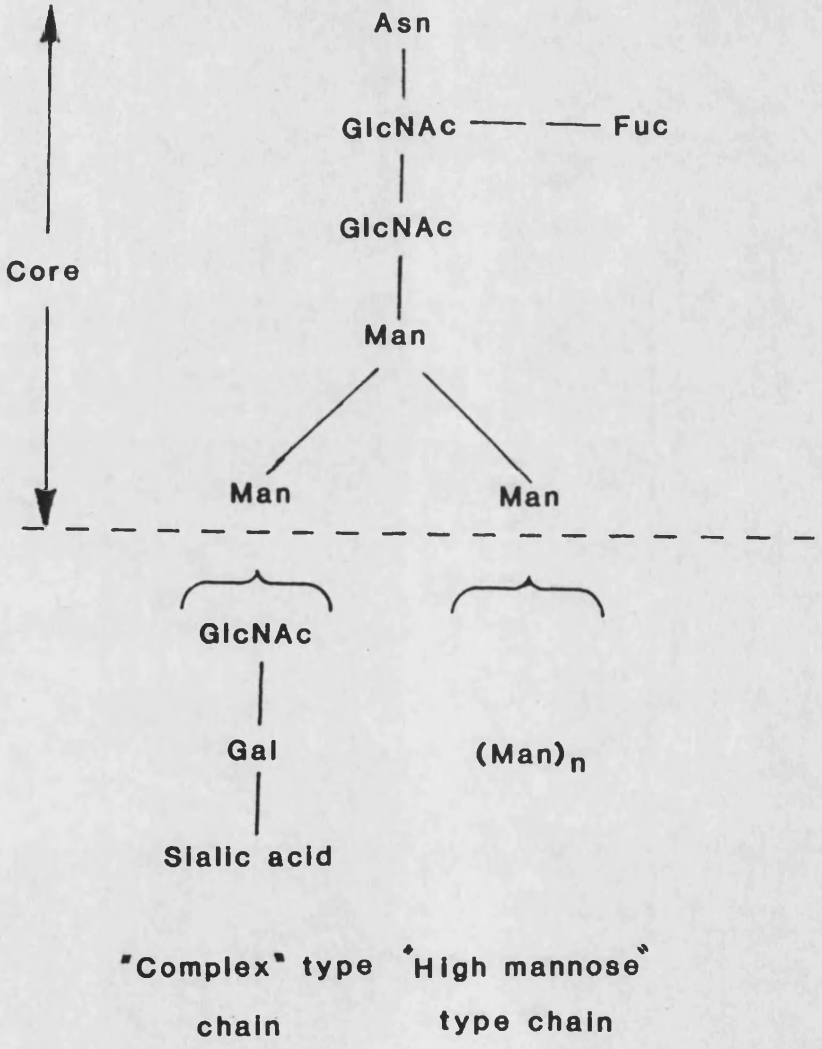


Figure 3. Schematic representation of a typical N-glycosidically-linked oligosaccharide in secreted glycoproteins. (After Montreuil, 1975)

their total sugar content and often their complete monosaccharide sequence can be revealed by a variety of chemical, enzymic or physical techniques (Table 1.3).

Overall Sugar Content

i) Total hydrolysis

The actual sugar content of a glycoprotein can be quite difficult to determine (Marshall and Neuberger, 1970). Colorimetric methods (Dische, 1962, Ashwell, 1966) have been used to estimate total carbohydrate in glycoproteins. The phenol-sulphuric acid method has been used to measure total hexose, while the cysteine-sulphuric acid method is more specific. Complete quantitative analysis requires liberation of the monosaccharide from their glycosidic linkages and identification, commonly by gas-liquid chromatography.

Hydrolysis is usually carried out under acid conditions. This is much more difficult for carbohydrates than for amino acids because monosaccharides generally vary greatly in their stabilities towards hot acid. Hexosamines are more resistant than most, but, even so, 5-15% of amino sugars such as N-acetylglucosamine and N-acetylgalactosamine are destroyed on heating in 4M HCl at 100^{o}C for 16h. Sialic acids are rapidly destroyed on heating with dilute mineral acids while aldoses such as mannose and

Table 1.3 Steps in the Sequence Analysis Glycans

1. Proteolysis: pronase, trypsin, chymotrypsin, collagenase, etc

2. Release of pure oligosaccharide chains: alkaline borohydride, endoglycosidases

3. Separation of glycopeptides: gel filtration, ion-exchange chromatography, paper electrophoresis and chromatography

4. Composition: gas-liquid chromatography (monosaccharides), ion-exchange chromatography (amino acids)

5. Carbohydrate sequence determination:
   a) chemical: methylation, periodate oxidation
   b) enzymic: exo- and endo-glycosidases
   c) physical: mass spectrometry, nuclear magnetic resonance

galactose, which do not contain nitrogen, occupy something of an intermediate position between the two extremes. The glycosidic linkage of amino sugars is protected by NH_3^+. Sialic acid residues are very readily removed from glycoproteins by hydrolysis with 0.05M H_2SO_4 at 80°C for 1h. Fucose is also easily removed from glycoproteins by mild acid treatment, whereas the galactopyranosides and glucopyranosides are 5 and 25 times (respectively) less readily hydrolysed than the 6-deoxyhexose.

Information concerning the nature of the carbohydrate linkage can also be obtained from hydrolysis. Two types of glycosidic linkages can be distinguished by treatment with mild alkalis. N-glycosidic linkages from N-acetylglucosamine to the amide nitrogen of asparagine, (Figure 2ß) are relatively stable to hydrolysis by strong alkali. O-Glycosidic linkages from N-acetylgalactosamine to the hydroxy group of serine or threonine (Figure 2ᾳ) can be cleaved by mild alkaline treatment which promotes a β-elimination reaction (Figure 4). If alkaline borohydride is used to free oligosaccharides from amino acid residues the reducing end of the oligosaccharide is protected as the alditol by reduction with the sodium borohydride (Figure 4). The production of N-acetylgalactosaminitol and 2-aminoacrylic acid (from serine) or 2-aminocrotonic acid (from threonine) is particuarly useful in determining the nature of the carbohydrate linkages (Sharon, 1975, Farrar

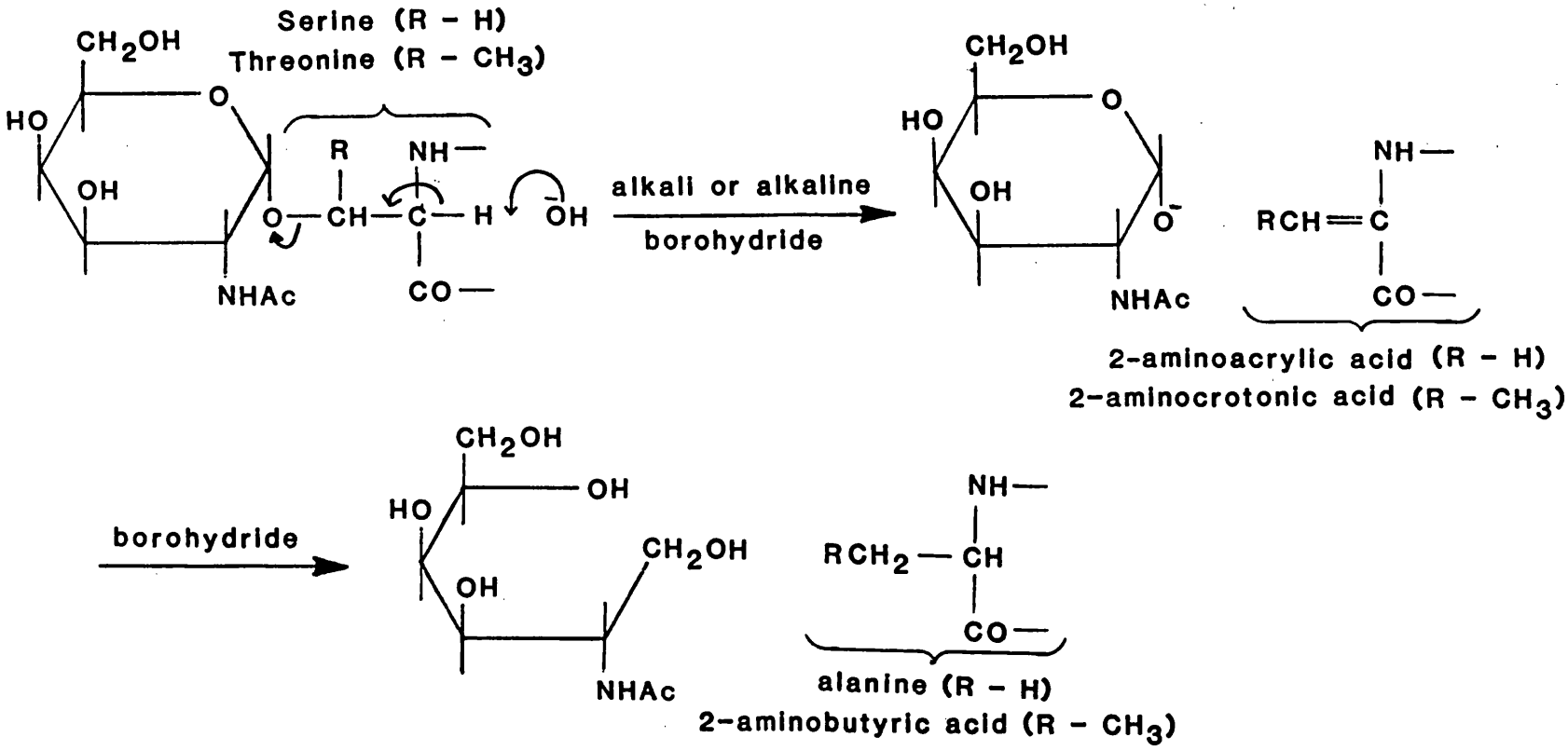


Figure 4. Scheme for Alkaline Borohydride Reduction

and Harrison, 1978).

ii) Gas-Liquid Chromatography

Gas-liquid chromatography can provide much quantitative and qualitative information on the monosaccharide content of a glycoprotein and hence has been extensively used for this purpose.

The first account of gas-liquid chromatography (GLC) was published by Martin and James in 1952. In 1957 Golay pioneered the use of coated capillary columns. The method provides a rapid and efficient means of partitioning compounds of similar structure, and when used in conjunction with flame ionisation detectors renders possible the analysis of very much smaller quantities of material than can be achieved by other techniques. These features were particularly desirable for the determination of the carbohydrate composition of glycoproteins because the large quantities of amino acid present generally interfere to a greater or lesser extent with most colorimetric assays.

In gas-liquid chromatography, a carrier gas passes through a column containing small particles which acts both as a support and a large surface area for a thin coating of liquid (liquid phase). Each component of a mixture will be partitioned between the mobile gas phase and the stationary liquid phase. However, as the gas is flowing, equilibrium is not established and the component

is carried along the column, being continually partitioned between the two phases. The absolute time that a component is retained on the column is difficult to determine because the actual time of injection and the time spent by the component between the column and detector are not easily ascertained. To overcome this problem (and several others) a reference compound is added to the sample and the retention times can be related to that for the reference. This reference compound is known as an internal standard.

Most quantitative analyses by GLC are carried out by the process of internal standardisation in which a known amount of internal standard is added to the sample at the beginning of the analytical procedure, any subsequent losses are assumed to affect both the standard and the sample in question to an equal extent. The peak area of the internal standard is taken into account in the calculations. Because of their tendency to char at high temperatures sugars must be derivatisated prior to their analysis by gas-liquid chromatography. Further details of GLC analysis can be found in reviews by Clamp et al (1972) and Dutton (1973, 1974).

Detailed Structural Analysis of Carbohydrate Structures

Detailed structural analysis of the carbohydrate structures of glycoproteins almost invariably necessitate the removal of all or most of the protein and the

purification of the resultant glycopeptide(s) or oligosaccharide(s). The carbohydrate component may be isolated by hydrolysis or exhaustive digestion of the macromolecule with a mixture of proteases and glycosidases (enzymes metabolising complex carbohydrates) or glycosidases alone. The enzymic treatment provides oligosaccharides with the minimum number of amino acid residues attached (Spiro, 1973, Sharon, 1975). The resulting glycopeptides may then be fractionated using gel filtration or ion-exchange chromatography (Kawasaki and Ashwell, 1976, Harrison et al., 1975, Harrison, 1977, Farrar and Harrison, 1978).

i) Glycosidases

Glycosidases are valuable tools with which to study structural features since these enzymes often display rather exact specificities for their substrates. The enzymes most widely used are the glycosidases which fall into two groups: the exo- and endo-glycosidases. Exo-glycosidases only cleave off sugars when the specific monosaccharide is a terminal residue. Most exo-glycosidases display great specificity for the sugar moiety and for the anomeric configuration of the glycosisic linkage. Stepwise removal of monosaccharides from a glycopeptide by exo-glycosidases followed by gas-liquid chromatography of the products can give a great deal of information on the carbohydrate structure.

Table 1.5 Endo-glycosidases

ENZYME	SOURCE	ENZYME SPECIFICITY
Endo-β-N-acetylglucosaminidase		
Type A		
D	Streptococcus pneumoniae	manα1→3manβ1→4glcNAcβ1↓→4glcNAc-Asn
H	Streptomyces plicatus Streptomyces griseus	manα1→3manα1→6manβ1→4glcNAcβ1↓→4glcNAc-Asn
CI and CII	Clostridium perfringens Fig Mammalian kidney, liver, spleen Hen oviduct	(1) manα1→3manα1→4glcNAcβ1↓→4glcNAc-Asn (2) manα1→4manα1↘ 6manβ1→4glcNAcβ1→4glcNAc-Asn manα1↗ ↑
Type B	Almond emulsin	manα1→4glcNAcβ1→4glcNAc↓-Asn
Endo- -N-acetylgalactosaminidase	Streptococcus pneumoniae Clostridium perfringens	galβ1→3galNAc linked to ser or thr*
Endo- -galactosidase	Streptococcus pneumoniae Escherichia freundii	glcNAcβ1→3galβ1↓→4glc(glcNAc)-R gal(galNAc)α1→3galβ1→4glcNAcβ1-R 2 ↑ 3 ↑ fucα1 (fucα1) 0 or 1

Type A enzymes all split the glycosidic linkage between the two *N*-acetylglucosamine residues immediately adjacent to the asparagine residue of the polypeptide. D and CI but not H or CII hydrolyse this bond even if the chitobiose sequence is substituted with fucose. The α3-substituted mannosyl unit at the non-reducing end of glycopeptides susceptible to D and CI must be unsubstituted. The enzymes also hydrolyse oligosaccharides. H requires a more complex sequence of mannosyl residues substituted onto the C(6) of the mannosyl residue of the trisaccharide core and does not react when this core is substituted with fucose.

Type B endo-β-N-acetylglucosaminidase is reactive with both intact glycoproteins and glycpeptides.

* The enzyme is inactivated by any substitution of this disaccharide.

R - hydrogen, sugars or ceramide.

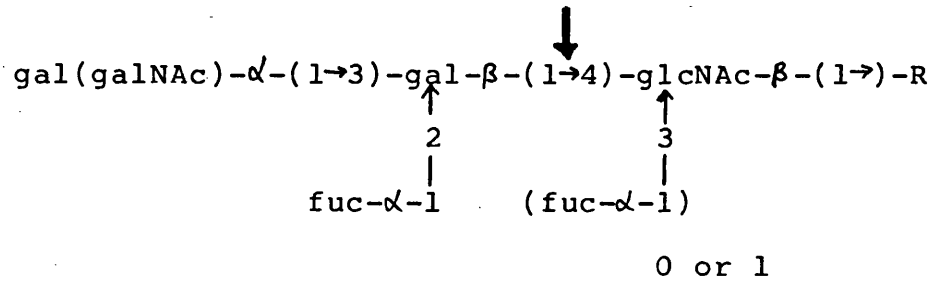
⬇ - point of hydrolysis.

(1) CI only

(2) CII only

Table 1.4 gives a list of a few of the many commercially available exo-glycosidases (from Hughes, 1983).

Endo-glycosidases are as valuable as exo-glycosidases in elucidating carbohydrate structures. They hydrolyse internal linkages and release oligosaccharises from glycoproteins or from glycopeptides. Since some of these enzymes react with intact glycoproteins and even the glycoproteins present on intact cells, their availability has also opened up the possibility of assessing, in a direct way, the biological roles of the carbohydrate moieties of glycoconjugates. For example, the Streptococcal endo-β-galactosidase hydroylses the branched oligosaccharides shown below.



R = hydrogen or sugar

⬇ - indicates point of hydrolysis

These structures represent the antigenic determinants of human blood groups A (with N-acetylgalactosamine terminal unit) or B (with galactose terminal unit). The N-acetyl-glucosamine is an essential part of the susceptible structures, gal-β-(1 4)-glcNAc likages but

Table 1.4 Exo-Glycosidases used for Determination of Carbohydrate Sequences in Glycoproteins (from Hughes, 1983)

Glycosidase	Source	Linkage Specificity
Neuraminidase	Streptococcus pneumoniae Clostridium perfringens	NeuNAcα2→3(6)gal, NeuNAcα2→6glcNAc
	Vibrio cholerae	NeuNAcα2→3gal>2→6
	Influenza virus	NeuNAcα2→3gal
Galactosidase (β)	Streptococcus pneumoniae Clostridium perfringens	Galβ1→4glcNAc only
	Jack bean meal Aspergillus niger	Galβ1→4glcNAc1→3
β-Mannosidase	Hen oviduct Turbo cornutus Snail Pineapple	Not defined
α-Mannosidase	Jack bean meal	Manα1→2man = α1→6>α1→3
β-Fucosidase	Clostridium perfringens Aspergillus niger	Fucβ1→2gal only
	Bacillus fulminans	Fucβ1→2gal only
	Turbo cornutus	All known linkages
	Charania lampas	
	Almond emulsin	Fucβ1→3(4)gal not α1→2 not Fucα1→6 glcNAc
β-N-Acetyl-glucosaminidase	Streptococcus pneumoniae Clostridium perfringens Jack bean meal	Broad

not gal-β-(1→3)-glcNAc likages are hydrolysed. Both these linkages are present in separate chains substituted with fucose and either galactose or N-acetyl-galactosamine to form distinct chains with A and B blood group activity. Streptococcus enzyme can discriminate antigenic structures and exhibits higher specificity than antibodies in this case (Hughes, 1983). More information about glycosidases can be found in the review of Hughes (1983). Table 1.5 lists some of the commercially available endo-glycosidases.

ii) Methylation Analysis

Chemical techniques are also widely used in addition to enzyme treatment for structural studies of the carbohydrate units of glycoproteins. Premethylation of free hydroxyl groups, followed by acid hydrolysis and gas-liquid chromatographic analysis of the partially methylated monosaccharides is one approach which has been extensively used. A technique developed by Hakomori (1964) results in complete methylation of all free hydroxyls as well as N-methylation of the acetamide group in hexosamine residues without loss of N-acetyl groups. This methylation is usually performed on isolated glycopeptides and rarely on intact glycoproteins. Gas-liquid chromatography of the methylation products can reveal a great deal about the structural organisation of the sugars of a glycoprotein. Fully methylated sugars

have a high volatility and even oligosaccharides have been separated in this way (Karkkainen, 1971).

iii) Nuclear magnetic resonance

Structural analysis of a complex carbohydrate such as that found in a glycoprotein has more recently been carried out by using such techniques as mass spectrometry and nuclear magnetic resonance spectroscopy.

^{1}H Nuclear magnetic resonance spectroscopy (^{1}H-n.m.r) has contributed significantly to the extension of knowledge on the structure and conformation of carbohydrates. However, this technique requires the availability of model compounds (Dorland et al., 1978a, b, Fournet et al., 1978) that have structures closely related to the complex carbohydrate being characterised. Nuclear magnetic resonance spectroscopy is an inherently insensitive technique and the richness of information contained in an nuclear magnetic resonance spectrum often limits the size and complexity of the molecules that can usefully be studied. Despite its apparent limitations, the structure of a homogeneous oligosaccharide can be revealed, in conjunction with methylation analysis, from as little as 2mg of material whereas much larger amounts are required for analysis by more convential chemical and enzymic techniques (Fournet et al., 1978). ^{13}C-n.m.r has advantages over ^{1}H when dealing with larger molecules such as polysaccharides, the spectra in the latter case

being often too complex.

iv) Mass spectrometry

Combined gas-liquid chromatography and mass spectrometry (GLC-MS) can be used to separate and identify permethylated oligosaccharide alditols containing several hexose units (Lundblad et al., 1980). The gas-liquid chromatography is efficient in the separation of the constituents of a mixture but does not always give full identification, whereas, the mass spectrometer can identify a single compound but is less efficient in the study of complex mixtures (Frigerio, 1974). The principle of mass spectrometry is that molecules of a substance, in a gaseous phase, are ioised and the ions produced are accelerated in an electric field. They are deviated in a magnetic field and then arrive at a collector generating a signal the intensity of which is proportional to the number of ions arriving.

Further information on mass spectrometry and nuclear magnetic resonance can be found in reviews by McNeil et al.,1982, Nilsson and Zopf, 1982, Barker et al., 1982 and Bock and Pedersen, 1983.

v) Fast atom bombardment (FAB)

Over the past two decades mass spectrometry has evolved to a method which is capable of providing both molecular weight and structural information on intact oligosaccharides, glycopeptides and glycolipids (for review see Reinhold and Carr., 1983). Various methods of mass spectrometry (e.g. electron ionization, chemical ionization and direct chemical ionization mass spectrometry) involve chemical manipulation to produce a relatively volatile derivative. Fast atom bombardment circumvents the requirement for chemical derivatization. Although molecular weight information can almost always be obtained by FAB, structurally significant fragment ions may be absent or of such low abundance that they are obscured by the background signals. FAB has other limitations in that sequence-specific fragments are often absent or in very low relative abundance in the FAB mass spectra of N-linked, high-mannose oligosaccharides and other neutral oligosaccharides. Enhancement of structural information obtained from FAB of carbohydrate structures has recently been achieved (Carr et al., 1985).

Lectins

Lectins (Lat.: Legere - to pick out or choose) also referred to as plant agglutinins, phytoagglutinins, phytohaemagglutinins, or haemagglutinins, represent a wide class of proteins or glycoproteins which are found predominantly in plants, particularly in the seeds of the leguminosae. It had been known for a long time that extracts of certain plant seeds agglutinate the erythrocytes of various animals. Stillmark (1888, 1889) was the first to describe the phenomenon of haemagglutination by plant extracts. These extracts were from the caster bean Ricinus communis. Soon after this discovery, Hellin (1891) found another lectin, abrin, present in the genus of Abrus precatorius. Already in 1891 these two lectins were used by Ehrlich in his classical studies on immunity which led to the discovery of the specificity of antibodies.

Following the discovery of the lectin from Ricinus, extracts of other plants were examined for haemagglutinating activity and a number of interesting lectins were discovered. However, up until the late 1940s lectins appeared to have no important scientific or commercial application. The independant studies of Renkonen (1948) and Boyd and Reguera (1949) led to the discovery of blood-group specific lectins. This discovery resulted in renewed interest, and in the

intervening years many other lectins have been discovered with and without blood-group specificity.

In 1980 Goldstein et al proposed the following definition:

1) A lectin is a sugar-binding protein or glycoprotein of non-immune origin which agglutinates cells and/or precipitates glycoconjugates.
2) Lectins should bear at least two sugar binding sites.
3) The specificity of a lectin should be defined in terms of monosaccharides (or simple oligosaccharides) that inhibit lectin-induced agglutination (or precipitation) reactions.

More recently, Kocourk and Horejsi (1981) added another proviso to the definition, viz: lectins should be devoid of enzymic activity towards sugars to which they bind and should not require free glycosidic hydroxyl groups on these sugars for their binding.

Mannose Binding Lectins

The lectin from Concanavalia ensiformis (Con A) is the most notable of the plant lectins. It was first isolated and crystallised by Sumner (1919). It is the most extensively investigated lectin, and the only one for which the primary structure has been established. In

1936 Sumner and Howell suggested that haemagglutination by Con A may be a consequence of the reaction of the lectin with carbohydrates in stroma proteins. In addition to its ability to agglutinate cells and to precipitate glycoproteins and polysaccharides, Con A possesses strong mitogenic activity. It is well established (Hardman and Ainsworth, 1972, Wang et al., 1971, Edmundson et al., 1971) that Con A consists of polypeptide subunits, of molecular weight 26,000. At pH 5.6 and below, two protomers are associated in a dimer of molecular weight 52,000. Above pH 5.6, the dimers aggregate, forming a tetramer. Con A also exhibits a reversible, temperature dependent dimer - tetramer transition (Gorden and Marquardt, 1974).

The metal ions Ca^{2+} and Mn^{2+} are required for carbohydrate-binding and there is one binding site for each ion (plus a saccharide) per subunit, (Yariv et al., 1968, Kalb and Levitzki, 1968). Both metals can be reversibly removed from the protein at low pH. In addition Mn^{2+} can readily be replaced by other transition metals, such as Ni^{2+} without any substantial change in carbohydrate-binding of the lectin (Agrawal and Goldstein, 1968). Binding of Mn^{2+} to demetallised Con A must precede Ca^{2+} binding (Hassing and Goldstein, 1970, Kalb and Levitzki, 1968). The binding of the transition metal appears to induce the formation of a specific Ca^{2+} ion-binding site (S2), (Kalb and Levitzki, 1968). Mn^{2+} and Ca^{2+} stabilise the conformation of the subunit, in

their absence, the four carboxyl groups clustered at the metal binding-sites would strongly repel one another (Hardman, 1973). Mn^{2+} and Ca^{2+} also seem to protect the protein against heat inactivation (Doyle et al., 1976) and against hydrolysis by proteolytic enzymes (Thomasson and Doyle, 1976). Further information on the lectin can be found in various reviews (Cunningham, 1975., Bittiger and Schebli, 1976., Goldstein and Hayes,1978).

Other lectins that have been shown to bind mannose include those from Lens culinaris (Lentil) and Pisum sativum (Pea). Hemagglutinating activity in the common lentil was first reported by Landsteiner and Raubitschek (1907). The lentil lectin has been isolated in a pure form and studied for its physical-chemical properties and interactions with carbohydrates (Howard and Sage, 1969., Ticha et al., 1970., Toyoshima et al., 1970., Howard et al., 1971., Paulova et al., 1971). The lectin from the pea was not purified until more recently (Huprikar and Sohonie, 1965., Shinohara, 1971., Betail et al., 1969., Guillot et al., 1969., Onodera and Shinohara, 1973., Trowbridge, 1974). Both lectins have been shown, like Con A, to have Mn^{2+} and Ca^{2+} bound to each molecule (Ticha et al., 1970., Entlicker et al., 1970).

On the basis of studies with various mono- and disaccharides, the pea lectin had been classified with lentil lectin and Con A as having α-glucosyl- and α-mannosyl-binding-specificity (Allen et al., 1976., Van Wauwe et al., 1975). In more recent years, the

carbohydrate-binding specificities of both lentil lectin (Kornfeld et al., 1971., Young and Leon, 1974., Toyoshima et al., 1972) and Con A (Kornfeld and Ferris, 1975., Baenziger and Fiete, 1979) have been examined using glycopeptides with oligosaccharide chains of varying structure to probe the sugar requirements for tight binding. It was concluded from these studies that both lectins, while requiring α-mannosyl groups, probably interact with multiple sugar residues rather than a single α-mannose residue to promote tight binding. Investigations by Kornfeld et al (1981) showed that the presence of a fucose residue attached to the asparagine-linked N-acetylglucosamine residue of a test glycopeptide was essential for high affinity binding to both pea and lentil lectin-Sepharose but not to Con A-Sepharose.

Galactose-Binding Lectins

Extracts of peanut (Arachis hypogaea) have been reported to agglutinate neuraminadase-treated human red blood cells (Bird, 1964., Uhlenbruck et al., 1969). The extract was found to have a high affinity towards the disaccharide Gal-β-(1→3)-GalNAc. Its binding activity was found to be inhibited by galactosides but not by free N-acetylgalactosamine. Peanut lectin has been purified (Lotan et al., 1975., Terao et al., 1975) by using affinity chromatography and has been found to have a

molecular weight of 110,000.

Peanut agglutinin has no covalently bound sugar. The lectins agglutinate erythrocytes of all human ABO blood types equally well, but only after they have been treated with neuraminadase. Of the monosaccharides tested for their ability to inhibit agglutination, only D-galactose and α- and β-D-galactosides were active. Highly inhibitory activity was found with the disaccharide D-Gal-β-(1→3)-D-GalNAc and with the desialylated glycoproteins: α_1-acid glycoprotein, fetuin, glycophorin and human blood group NN or MM antigen (Lotan et al., 1975). There have been conflicting reports about the mitogenicity of the peanut lectin towards human peripheral blood lymphocytes (Novgrodski et al., 1975., Terao et al., 1975).

Another major galactose-binding lectin is that obtained from Ricinus communis and already mentioned briefly. Extracts from Ricinus contain two, chemically distinct, carbohydrate-binding proteins: a toxin and a haemagglutinin (Takahashi et al., 1962., Ishiguro et al., 1964). The haemagglutinin is separable from ricin (the toxin) by selective elution of ricin from Sepharose with 2-acetamido-2-deoxy-D-galactose followed by elution of the haemagglutinin with D-galactose (Nicolson et al., 1974). The toxin (ricin) consists of two peptide chains of about equal size, the A-chain and the B-chain. As a note of interest, the toxicity of ricin may result from the binding of the toxin to mammalian cells by way of its

B-subunit (presumably through a carbohydrate structure) followed by ingestion of toxin (A-subunit) and finally, by A-chain inhibition of protein synthesis (Olsnes and Pihl, 1976).

The agglutinin from Ricinus contains about 12% (by weight) of carbohydrate, which is not required for binding. The failure of EDTA to inhibit R.communis agglutinin suggests its lack of dependence on metal cations for its activity (Lis and Sharon, 1973). Sugars of the D-galactopyranose configuration are bound most effectively with some preference for β-D-galatosides, although the anomer is also bound. Lactose is three times as effective at inhibiting haemagglutination as is methyl-β-D-galactopyranoside.

N-Acetylglucosamine-Binding Lectins

Triticum vulgaris lectin is not a blood group-specific haemagglutinin. It will agglutinate all types of human erythrocytes, as well as a variety of normal and neoplastic animal cells. The agglutination of cells is inhibited by N-acetylglucosamine and its β1→4 oligomers (Burger and Goldberg, 1967., Allen et al., 1973). In addition, T.vulgaris lectin has been claimed to bind N-acetylneuraminic acid on the basis of equilibrium dialysis and nuclear magnetic resonance data (Greenaway and LeVine, 1973., Jordan et al., 1977). Furthermore, the agglutination of neuraminidase-treated

cells usually requires a higher concentration of T.vulgaris lectin than that required for untreated cells (Burger and Goldberg, 1967., Nicolson et al., 1975)

Monsigny et al (1980) showed that N-acetyl-neuraminic acid, gangliosides and glycoproteins containing N-acetylneuraminic acid do bind to T.vulgaris lectin under specific conditions. The specific binding of N-acetylneuraminic acid to wheat-germ agglutinin is based on configurational similarities between N-acetylneuraminic acid and N-acetylglucosamine.

The interaction between T.vulgaris lectin and glycoconjugates containing N-acetylneuraminic acid has been shown to be dependent on a charge effect. Succinylated T.vulgaris lectin which is negatively charged at physiological pH, in contrast to T.vulgaris lectin which is positively charged, does not bind cell surface glycoconjugates containing N-acetylneuraminic acid but does bind cell surface glycoconjugates containing N-acetylglucosamine (Monsigny et al., 1980).

N-Acetylgalactosamine-Binding Lectins

The lectin from Glycine max (Soybean) is a glycoprotein containing approximately 7% (by weight) of carbohydrate. It is a metalloprotein containing Ca^{2+} and Mn^{2+} which is inactivated by Al^{2+}, Fe^{2+} and Pb^{2+}. The carbohydrate-binding specificity of soybean agglutinin appears to be directed towards both anomers of

N-acetylgalactosamine. By inhibition of haemagglutination, Lis *et al*. (1970) found that four disaccharides, in which N-acetylgalactosamine was linked β-D-(1→6), α-D-(1→3), β-D-(1→3), and β-D-(1→4), respectively, to D-galactose, were approximately equivalent to N-acetylgalactosamine inhibition. From other investigations, Pereira *et al*. (1974) concluded the following: Firstly, the lectin exhibited greatest affinity for N-acetylgalactosamine, its glycosides, and oligosaccharides in which this was the non-reducing, terminal, sugar group (the reaction was inhibited to a lesser extent by D-galactose and its derivatives). Secondly there is a slight preference for α- over β-glycosidically linked sugars. Finally, substitution of blood-group A-active oligosaccharides by L-fucosyl residues greatly diminishes their susceptibility to haemaglutination by the lectin from *G.max*. The agglutinin from *G.max* is unusual among the lectins in that haemagglutination is inhibited most effectively by the monosaccharide rather than by oligosaccharide structures.

L-Fucose-Binding Lectins

The blood-group H(O)-specific haemagglutinating activity of *Lotus tetragonolobus* was originally reported in 1948 (Renkonen, 1948). Morgan and Watkins (1953), in agreement with the results of Renkonen (1948), found that the lectin from *L.tetragonolobus* agglutinated type O

cells considerably better than it did A_2 cells, whereas types A_1, B and AB were not agglutinated by this lectin. Furthermore this agglutination could be inhibited by purified H(O)-active blood-group substance, and by L-fucose.

Investigations by Pereira and Kabat (1974) showed that precipitation of human blood-group H substance by L.tetragonolobus lectin could most effectively be inhibited by an H-active difucosyl oligosaccharide.

A second fucose-binding lectin, from Ulex europeus, has become a standard serological reagent (Race and Sanger, 1975). There are two lectins of distinct sugar-binding specificity in Ulex extracts. Ulex I is readily inhibited by L-fucose derivatives while Ulex II binds β-D-glucosides.

A Sialic Acid-Binding Lectin

A sialic acid-binding lectin has been isolated from horse-shoe crab (Limulus polyphemus) haemolymph. The Limulus lectin agglutinates erythrocytes (Cohen et al., 1974). Calcium ions are required for, and sometimes enhance, the agglutination reaction (Roche and Monsigny, 1974). N-acetylneuraminic acid and D-glucuronic (but not D-galacturonic) acid specifically inhibit agglutination of horse erythrocytes by Limulus lectin (Nowak and Barondes, 1975). Precipitin reactions were found to be inhibited by bovine submaxillary-mucin but not by

desialated mucin (Oppenheim et al., 1974) and these studies indicate that the Limulus polyphemus lectin possesses the capacity to react with biopolymers and cells containing terminal sialic acid residues.

The sugar and blood group specificities of various lectins can be seen in Table 1.6.

Table 1.6 Sugar Specificity and Blood Group Requirement of Various Lectins

LECTIN	SUGAR	BLOOD TYPE
Lotus tetragonolobus	α-L-Fucose	O
Ulex europeus	α-L-Fucose	O
Limulus polyphemus	Fetuin, Glucuronic acid	Horse
Triticum vulgaris	N-Acetyl-D-glucosamine	A
Pisum sativum	NAcGlc, α-D-Glc, D-Mannose	A
Lens culinaris	NAcGlc, α-D-Glc, D-Mannose	A
Glycine max	NAcGal, Lactose	A
Arachis hypogaea	D-Galactose (Desialated) β-D-Gal-D-GalNAc	A

Aims of the Project

Although sugars constitute only a small percentage to the total weight of the acetylcholine receptor their role in the glycoprotein may be much more important than present research indicates. Carbohydrates have been implicated in the antigenicity of a number of glycoproteins (Fiezi, 1981, Uemura et al., 1983), in fact, the binding of antibodies from myasthenic serum to purified T.californica acetylcholine receptor has been shown to be completely inhibited by 50mM N-acetylglucosamine (Hall et al., 1983). These results suggest that the antibody recognises a glycosyl side chain on the acetylcholine receptor. If this is indeed the case then knowledge of the subunit distribution of sugars and oligosaccharide structure of the receptor may be of great importance in the understanding of diseases such as myasthenia gravis.

The introduction to this thesis gives the impression that there has been substantial investigation into the carbohydrate part of the nicotinic acetylcholine receptor. However, most investigations have simply indicated the presence of various sugars without giving much indication as to the quantity or subunit distribution.

One of the major problems with ascertaining the structure of an oligosaccharide is the relatively large amounts of material required. Even using nuclear

magnetic resonance at least 2mg of pure oligosaccharide is required. This limits the number of analytical techniques that can be employed in determining the saccharide structure of the acetylcholine receptor as only relatively small amounts of intact glycoprotein can be purified at one time. An additional problem is that each subunit may have a different sugar content thus complications arise when looking at the whole receptor molecule.

Using gas-liquid chromatography, a relatively cheap and common method of analysis, coupled with enzyme treatment with glycosidases, the quantitative and qualitative carbohydrate content of the acetylcholine receptor was determined. Additional information on the subunit distribution and oligosaccharide structure of the receptor has also been gained from various techniques using lectins. The lectins bind with high specificity to particular saccharide structures and thus only very small amounts of the glycoprotein were required for analysis.

2 MATERIALS

Torpedo marmorata were obtained from Institut de Biologie Marine, Arcachon, France, and the electric organs were stored at -80°C.

2.1 Buffers and Solutions

These were prepared by using glass distilled water and whenever possible AnalaR grade biochemicals. Buffers were made as described in Data for Biochemical Research (Dawson et al eds), 2nd Ed., 1974, University Press, Oxford, Great Britain.

2.2 Chemicals

^{125}I Na was purchased from the Radiochemical Centre., Amersham, Bucks, U.K. Benzoquinonium chloride was a gift from the Sterling Winthrop Research Institute., Renssalaer, New York, U.S.A. Lectins were obtained from Sigma Chemical Co.Ltd., Kingston-upon-Thames, Surrey, U.K. Other chemicals were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. Chromic chloride was purchased from BDH Chemicals, Poole, Dorset.

Ampholines were obtained from L.K.B Ltd., Croydon, U.K.

2.3 Chromatographic Media

Sephadex (G50 medium, G25 medium) and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Carboxymethyl Cellulose (CM 32), Carboxymethyl Sephadex (C-50), Diethylaminoethyl Cellulose (DEAE cellulose, DE52) and Cellulose Phosphate (P11) were obtained from Whatman Biochemicals Ltd., Maidstone, U.K. ACA 34 Ultragel was purchased from LKB Ltd., Croydon, U.K.

2.4 Snake Venoms and αToxins

Naja naja siamensis and *Bungarus multicinctus* venoms were purchased from Miami Serpentarium, Miami, Florida, U.S.A. Bungarotoxin (αBGT) was obtained from Boehringer Corp., Lewes, Sussex, U.K.

2.5 Spot test and Electrotransfer

Nitrocellulose paper (0.45μm) was purchased from Bio-rad Labs., Richmond, California U.S.A. Gelatin was obtained from Difco Laboratories, Michigan, U.S.A.

2.6 Enzymes

Neuraminadase (*Clostridium perfringens*) was purchased from the Boehringer Corp., Lewes, Sussex, U.K.

β-Galactosidase (E.coli) was obtained from the Sigma Chemical Co. Mixed Endoglycosidases D (D.pneumoniae) were purchased from Seikagaku Kogyo Co.Ltd., Tokyo. 103. Japan. Trichomonas foetus enzymes were generously provided by Dr. M.G. Ormerod, Institute of Cancer Research, Royal Cancer Hospital, Sutton, Surrey, U.K.

2.7 Gas Chromatography

D-Galactose, D-mannose, D-glucose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, L-fucose and Perseitol were obtained Sigma Chemical Co. Microflux vials were from Pierce Chemical Co., Rockford, Illinois, U.S.A All other reagents were AnalaR grade whenever possible.

The prepacked gas chromatographic columns were purchased from Perkin-Elmer Ltd., Bucks, England.U.K. The columns were conditioned before use as follows:- The detector end of the column was disconnected and the carrier flow rate set to 10 ml/min, the column was purged at room temperature for approximately 2h and the temperature was programmed ($1^{o}C$/min) up to 25 - $50^{o}C$ below the maximum recommended temperature.

2.8 Immunological Reagents and Red Blood Cells

Rabbit anti-(αBGT) serum was a gift from Miss J.Whyte; sheep and rabbit anti-(Torpedo AChR) antibodies

were supplied by Dr S. Wonnacott; monoclonal anti-(AChR) antibodies were provided by Miss S. Walsh (all of Biochemistry Department, University of Bath). The following sterile media and supplements were purchased from Flow Laboratories., Ayrshire, Scotland: Foetal calf serum, Minimum Essential Medium (M.E.M) (Eagle), Hepes 1M, 7.5% (w/v) sodium bicarbonate, L-glutamine (200mM), Penicillin - Streptomycin (5000 IU/ml and 5000 μg/ml, respectively).

Sheep red blood cells (SRBC) in Alsevers buffer were purchased from Tissue Culture Services., Slough, Berks. Human red blood cells (HRBC) were donated by departmental personnel. Guinea pig complement was obtained from Miles, Slough, Berks. Microtitre trays were purchased from Nunc, Gibco, Uxbridge, U.K. Bioeads, Sm2 were from Bio-Rad Labs, Richmond, C.A.

2.9 Hepes-Buffered Eagle Medium (H-Eagle)

Component	ml/100ml
M.E.M (10 x concentrated)	10
Hepes (1M)	2
L-Glutamine	1
Penicillin - Streptomycin	4
7.5% (w/v) sodium bicarbonate	1.13

Double distilled water to 100ml

Adjust pH with 1M NaOH to 7.4 - 7.6

3 METHODS

3.1 BUNGAROTOXIN STUDIES

3.1.1 Purification of α-Toxin from Bungarus multicinctus Venom

The α-toxin (αBGT) from Bungarus multicinctus was purified according to the method of Lee et al (1972). Bungarus venom (50mg) was dissolved in 50mM ammonium acetate buffer pH 5.0 (5ml) and applied to a CM Sephadex column (2.0cm x 25cm) previously equilibriated with the same buffer. The column was eluted with a linear gradient (340ml) of 0.05M (pH 5.0) - 0.5M (pH 7.0) ammonium acetate buffer. The flow rate was 15ml/h and fractions of 3ml were collected. Elution from the column was monitored at 280nm.

Peak II was pooled, lyophilised and redissolved in 0.05 M ammonium acetate pH 5.0 and applied to a CM Cellulose (CM 52) column (0.8cm x 14cm). The column was eluted with a linear gradient (200ml) of 0.05M (pH 5.0) - 0.2M (pH 7.0) ammonium acetate. The flow rate was maintained at 9.3ml/h and was continuously monitored at 280nm. Fractions of 1.4ml were collected.

3.1.2 Repurification of Commercial α-Bungarotoxin

Commercial αBGT (10mg) was dissolved in 0.05M

ammonium acetate, pH 5.0, 1ml and applied to a column of CM cellulose. The column was extensively washed with the same buffer and the αBGT was eluted with a linear gradient of 0.05M (pH 5.0) - 0.5M (pH.7.0) ammonium acetate. The flow rate was maintained at 10ml/h with a peristaltic pump and fractions of 1ml were collected. The fractions were assayed for protein and the main protein peak was pooled and lyophilised.

3.1.3 Iodination of Proteins

αBGT was labelled with ^{125}I by the chloramine T method of Hunter (1978). The procedure was as follows:- carrier-free ^{125}I (1mCi) was added to 50mM potassium phosphate buffer, pH 7.5, (10µl) in a plastic tube. The protein (5-10µg) in buffer (10-25µl) was added to the mixture and stirred. Chloramine T (50µg) in buffer (10µl) was added to initiate iodination. The reaction was carried out at 20°C for 60 sec with stirring. Termination of the reaction was achieved by adding sodium metabisulphite (120µg) in 50mM phosphate buffer (0.75ml), followed by KI (2mg) in the same buffer (200µl).

Separation of free from bound ^{125}I was achieved by gel filtration on Sephadex G25 (see chromatography section 3.1.5). Fractions corresponding to the protein peak were pooled and stored at 4°C.

Incorporation of ^{125}I into void volume peak (μCi/μl) =

$$\frac{\text{cpm (pooled tubes)}}{\text{cpm (total } ^{125}\text{I eluted)}} \times \frac{\text{mCi } ^{125}\text{I added}}{\text{total volume void vol. (ml)}}$$

Protein concentration in pool (pmol/μl) =

$$\frac{\text{cpm (pooled tubes)}}{\text{cpm (protein peak)}} \times \frac{\text{pmoles protein added}}{\text{total vol pool (ml) x 1000}}$$

3.1.4 Alternative Methods for Iodinating α-Bungarotoxin

Two alternative methods of iodinating αBGT were investigated in addition to the chloramine T method of Hunter (1978). These included the oxidative iodine monochloride method (Vogel et al., 1972) and a modification of this method (Doran and Spar, 1980). These methods are described below.

1) Oxidative Iodine Monochloride Method for Iodinating α-Bungarotoxin (Vogel et al,. 1972)

αBGT was labelled with ^{125}I by a modification of the methods of McFarlane (1958) and Helmkamp (1967) as follows: $Na^{125}I$ (1mCi) was incubated with a solution of 10mM iodine monochloride, 33mM hydrochloric acid and

170mM sodium chloride (7µl in total) at room temperature in a plastic tube. After 4 min, 0.4M NH_4Cl, pH 8.9, (13µl) and αBGT (2.5 nmoles) were added and the mixture was incubated for 2 min over ice. The reaction was stopped by the addition of 0.1M sodium metabisulphite (20µl). The mixture was diluted with 0.1M sodium iodide (20µl) and 10mM potassium phosphate buffer (930µl). Free iodine was separated from that bound to toxin by gel filtration on Sephadex G25 (see section 3.1.5).

2) Modification of the Oxidative Iodine Monochloride method (Doran and Spar, 1980)

The method of Helmkamp (1967) was modified by Doran and Spar (1980) for the labelling of microgram amounts of protein in a reduced reaction volume. The chemical basis of the oxidative ICl method is the oxidation of radioiodide (and stable iodine) to iodine monochloride which then iodinates the protein. The reaction generating the ICl moiety has been summarised by Helmkamp as:

$$2I^- + IO_3^- + 6HCl = 3ICl_3^- + 3H_2O$$

Calculations

$N = P \times (I/P)$

N = number of moles of ICl needed

P = number of moles of protein to be iodinated

I/P = desired maximum iodine to protein ratio

For every 3 moles of ICl produced, 2 moles come from iodide and one from iodate. Hence the number of moles of iodide needed (L) is

$L = (2/3) \times N$

As not all the iodide would, in most experiments, be supplied by radioiodide, the radioiodide is usually supplemented with stable iodide. ^{125}I has a molar specific radioactivity of 2.2 mCi/nmole. Let S be the desired molar specific radioactivity (mCi/nmole) of the iodinated protein. Then the number of moles of radioiodide (R) needed is:

$R = S \times P/(2.2\ mCi/nmole)$

Stable iodide (in the form of KI) = L - R

Iodate is supplied in excess (5 x L moles)

Reagents were added to a plastic disposable test tube in the following order:

Distilled water (100µl) containing (L - R) moles KI

$Na^{125}I$ stock (10 - 100µl) containing R moles of radioiodide.

5M NaCl (100µl)

Distilled water (100µl)

Distilled water (100µl) containing 5 x L moles KIO_3

250mM HCl (100µl)

The contents of the tube were briefly mixed and incubated at room temperature for 5 min after which time they were added to a solution (20µl) ofαBGT in 75mM NaOH in borate buffered saline. After 1 min, 10mM potassium phosphate buffer (1ml) containing 2% BSA was added and the free ^{125}I was separated from the radiolabelled toxin by gel filtration as before.

3.1.5 Gel Filtration of Iodinated α-Bungarotoxin

Free ^{125}I was separated from iodinated αBGT by gel filtration on Sephadex G25. The iodination mixture was applied to a column (1.5cm x 25cm) of Sephadex G25 (medium) which had been previously swollen and packed according to the manufacturers instructions. Elution was carried out with 10mM sodium phosphate buffer, pH 7.5, containing 1% BSA. Chromatography was performed under gravity and fractions (1ml) were collected by hand. Figure 3.1.1 shows a typical example of the elution profile of iodinated αBGT on Sephadex G25.

3.1.6 Assay of Acetylcholine Receptor

Acetylcholine receptor was quantified in terms of

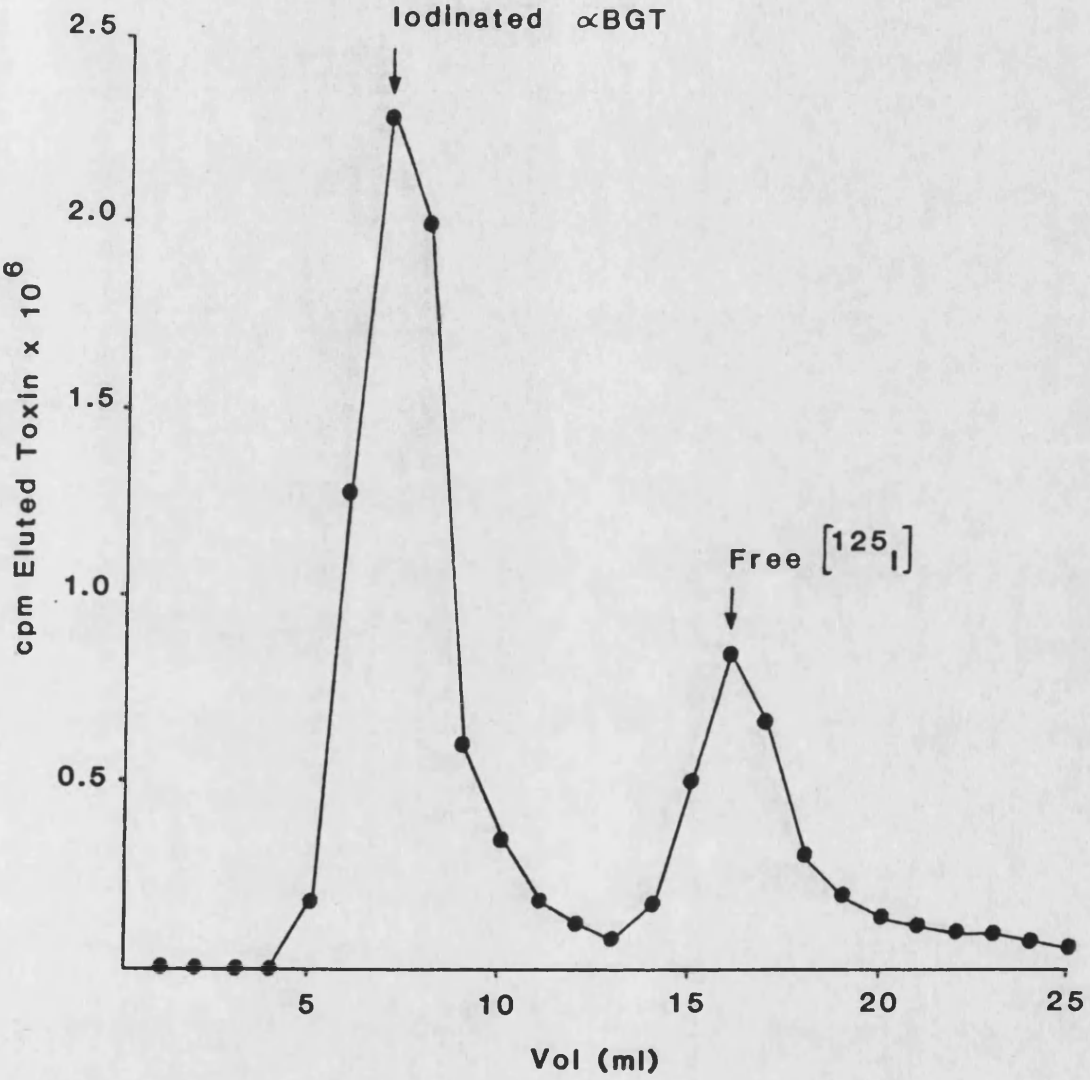


Figure 3.1.1 Elution profile of iodinated αBGT on Sephadex G25 column (1.5cm x 25cm). The column was eluted (under gravity) with sodium phosphate buffer, 10mM pH 7.5, containing 1% (w/v) BSA. Fractions of 1ml were collected and samples (10μl) counted for radioactivity.

α-bungarotoxin binding sites, different methods being used for purified and crude preparations.

i) Assay of purified Torpedo acetylcholine receptor was carried out by a modification of the method of Schmidt and Raftery (1973). All dilutions were made in 10mM potassium phosphate buffer, pH 7.4, containing 0.1% (w/v) BSA, 0.1% (v/v) Triton X100 and 0.02% (w/v) sodium azide (toxin binding assay buffer) unless otherwise stated. Duplicate dilutions of AChR (100μl) were incubated with ^{125}I-αBGT (50μl, approx. 10nMolar) for 60-90 min at 20^{o}C or overnight at 4^{o}C. The samples (diluted with toxin binding assay buffer, 1ml) were applied to a double thickness of moistened DEAE 81 - cellulose discs and filtered under suction. Tubes were washed with buffer (2 x 1ml), and the filter discs with a further 1ml. Bound radioactivity was counted in an LKB 1280 Ultrogamma counter. Non-specific binding was determined by including 25mM benzoquinonium chloride (10μl) and carrying out the assay as described before.

ii) Assay of crude Triton X100 solubilised AChR was carried out by precipitation with ammonium sulphate. Samples (100μl, diluted in toxin binding assay buffer) were incubated with ^{125}I-αBGT (50μl, approx. 10nMolar) for 1h at 20^{o}C after which time saturated ammonium sulphate solution (133μl) was added and the mixture was left overnight at 4^{o}C. The samples, diluted in 40%

ammonium sulphate (1ml), were applied to a GFC filter and washed as above by using 40% ammonium sulphate. Non-specific binding was determined by incubation in the presence of 10mM benzoquinonium chloride (50µl). Bound radioactivity was counted as before.

3.1.7 Antigenic Activity of ^{125}I-α-BGT

^{125}I-αBGT (0.52pmol in 100µl) was incubated with rabbit anti-(α-bungarotoxin) antiserum (5µl, dilutions ranging from 1/2 - 1/40) for 24h at 4°C. Non-specific binding was determined by preincubating immune serum with 0.1mM native toxin (5µl) (purified and unpurified commercial αBGT) for 1h at room temperature, or by substitution with non-immune serum. Toxin-antibody complexes were precipitated by the addition of sheep anti-(rabbit IgG) serum (120µl) followed by incubation at room temperature for 2h. The complexes were pelleted by centrifugation at 3000g for 10 min in an M.S.E. Mistral centrifuge. The pellets were washed twice with 10mM potassium phosphate buffer, pH 7.4, containing 0.15M NaCl, and 0.1% (w/v) sodium azide, and the radioactivity determined.

Immunobiological activity can be defined as

$$\frac{\text{Toxin-Ab complex ppt. (cpm) - nonspecific component (cpm)}}{\text{Total toxin added (cpm)}} \times 100$$

3.1.8 Biological Activity of Iodinated α-Bungarotoxin Preparations

Preparations of radiolabelled toxin (0.1 - 1.0 pmol, 50µl) were incubated with a 1000 fold molar excess of Torpedo AChR (100µl) for 90 min at room temperature, filtered through DEAE-cellulose filters to remove free ^{125}I-αBGT and the radioactivity was counted as above (methods 3.1.6).

Biological activity (%) can be defined as

$$\frac{\text{Labelled toxin bound (cpm)}}{\text{Total labelled toxin added (cpm)}} \times 100$$

3.1.9 Fractionation of Iodinated α-Bungarotoxin

αBGT was iodinated by the oxidative iodine monochloride method of Vogel et al (1972) and separated from free ^{125}I as described previously. The resultant

^{125}I-αBGT peak was loaded onto a column of CM Sephadex (C50) previously equilibriated with 10mM potassium phosphate buffer, pH 7.4, containing BSA (2mg/ml). Elution of the iodinated αBGT fractions was carried out with a linear gradient of the phosphate buffer (50ml) and the same buffer containing 80mM NaCl (50ml). The column was eluted at 15ml/h and fractions (1ml) were collected. Samples (10µl) were counted.

3.1.10 Competition Studies

Competition studies were used as an alternative method to that described in section 3.1.3 for determining the concentration of iodinated αBGT. There were carried out as follows. Aliquots (10µl) of iodinated αBGT preparations were incubated with 0 - 0.4 pmol of purified Torpedo acetylcholine receptor (100µl) and 0 - 10 pmol native αBGT (10µl) at 4^{o}C overnight. The tubes were filtered and counted as in method 3.1.6(i).

3.2.1 Purification of α-Naja Toxin

α-Naja toxin was purified from the lyophilised venom of Naja naja siamensis according to the method of Cooper and Reich (1972).

Venom (1g) was dissolved in distilled water (10ml) and applied to a column of cellulose phosphate (2.2cm x 34cm) packed according to the manufacturers instructions

and previously equilibriated with 10mM potassium phosphate buffer, pH 6.0. The column was washed with the same buffer until the absorbance of the eluant (monitored at 280nm with a Cecil Spectrophotometer) had returned to baseline. Elution was carried out with a linear gradient of potassium phosphate buffer pH 6.0 with 0.01M and 0.5M as limiting concentrations (800ml of each). The column was eluted at 60ml/h and fractions (15ml) were automatically collected. The peak protein fractions (OD 280 nm) were assayed for their ability to inhibit ^{125}I-αBGT binding to AChR. Aliquots of the peak fractions (instead of benzoquinonium chloride) were coincubated with purified AChR and αBGT as described in methods 3.1.6(i). Those fractions showing peak inhibition were pooled and ammonium sulphate added to give a saturated solution. The solution was allowed to precipitate overnight at 4^{o}C with stirring and then centrifuged at 30,000g for 10 min to isolate the protein. The pellets were dissolved in, and extensively dialysed against, distilled water and then lyophilised. The purified α-Naja toxin was stored at 4^{o}C.

3.2.2 Coupling of α-Naja Toxin to Sepharose 4B

Sepharose 4B was activated by the cyanogen bromide method of March et al (1974). Packed Sepharose 4B beads (50mls) were washed in 0.1M sodium chloride (1l) followed by water (500ml). They were then suspended in ice-cold

water (50-100ml) and stirred over ice with 2M sodium carbonate (100ml). Activation was carried out by mixing with cyanogen bromide (2.5g, 2g/ml in acetonitrile) for 2 min followed by rapid filtration and washing with ice-cold water.

α-Naja toxin (25mg in 0.2M sodium bicarbonate buffer, pH 9.4, 100ml) was coupled to the beads by stirring overnight at 4°C. The beads were then filtered, washed with water (400ml) and subsequently stirred with 1M glycine, pH 9.0 (200ml overnight at 4°C) to block unbound sites. The beads were finally washed alternately in 0.1M sodium acetate buffer, pH 4.0, containing 1M NaCl and 0.1M sodium borate buffer, pH 8.0, containing 1M NaCl. The affinity beads were stored at 4°C in 10mM potassium phosphate buffer, pH 7.4, containing 0.1% Triton X100 and 0.02% sodium azide.

The efficiency of binding was consistently good with more than 95% of the added protein being bound.

3.3 PURIFICATION OF TORPEDO MARMORATA ACETYLCHOLINE RECEPTOR

3.3.1 Purification of Acetylcholine Receptor using an α-Naja Toxin Affinity Column

Purification of Torpedo AChR was carried out (essentially as described by Lindstrom, 1976) by homogenising the frozen electric organs in 0.4M NaCl, pH 7.4, in a Sorvall Omnimix for 3 min at full speed, (50ml buffer to 50g frozen tissue). The homogenate was then centrifuged at 100,000g in a Beckman L5-65 centrifuge (S35 rotor) for 30 min at 4°C. The pellets were resuspended (with a Polytron) in 20mM potassium phosphate buffer, pH 7.4 (1g original tissue/ml) and centrifuged as before. The acetylcholine receptor was solubilised from the pellets in 50mM potassium phosphate buffer, pH 7.4, containing 1% Triton X100 (50ml to 100g original tissue) for 4h at 20°C or overnight ot 4°C after which time the extract was centrifuged at 100,000g in a Beckman L5-65 centrifuge (S35 rotor) for 1h at 4°C.

Purification of the solubilised acetylcholine receptor was achieved by stirring the Triton X100 extract with the α-naja toxin affinity gel (50ml) for 4h at 20°C or overnight at 4°C. The gel was then poured into a glass column (the non-bound fraction was collected and assayed for toxin binding activity, methods 3.1.6(ii))

and washed with 10mM potassium phosphate buffer, pH 7.4, containing 0.1% Triton X100 and 0.5M NaCl (500ml) and then with the same buffer minus the salt. The receptor was eluted from the gel by using 1M Carbamoyl choline (carbachol). Elution was achieved by mixing the gel with the carbachol overnight at $4^{o}C$. The gel was then poured back into the column and the carbachol washed off with 10mM phosphate buffer, pH 7.4, 0.1% Triton X100. The carbachol solution and washings were combined and dialysed against 10mM potassium phosphate buffer, 0.1% (v/v) Triton X100 (dialysis buffer).

The dialysed, affinity purified AChR was concentrated by passage through a column (3.5cm x 3cm) of DEAE cellulose (DE52). The bound receptor was washed with dialysis buffer (1l) and eluted with 0.5M NaCl in the same buffer (50ml). The column was eluted under gravity and fractions of 1ml were collected by hand and assayed for toxin binding (section 3.1.6(i)).

Precautions were taken throughout to prevent bacterial contamination and proteolytic degradation by including, in all buffers, the inhibitors listed in table 3.3.1. The purified acetylcholine receptor was stored at $4^{o}C$.

Table 3.3.1 Bacteriocides and Protease Inhibitors

1) 0.02% (w/v) sodium azide

2) 5mM EDTA

3) 0.5mM PMSF

4) 0.1mM Benzethonium chloride

5) 0.5g/l Bacitracin

3.3.2 Modifications of the Basic Method of Purification of Acetylcholine Receptor

The original method of purification was modified in two major ways. Both alternative methods were carried out omitting the second 100,000g centrifugation step. Solubilisation was carried out immediately after the first spin.

The first alteration was the substitution of the 1% solution of Triton X100 used for solubilisation of the receptor with 2% Triton X100. The second alteration involved the elution of AChR from the affinity gel by using 4mM benzoquinonium chloride (Lindstrom *etal*, 1981). This was achieved by recycling the benzoquinonium chloride solution through the affinity gel and the DEAE cellulose column for 18 - 24h at $4^{o}C$. One advantage with this method is that it avoids the need for dialysis to remove carbachol, thus shortening the purification time.

3.3.3 Coupling of Anti-(Torpedo AChR) Monoclonal Antibodies to Sepharose 4B

Monoclonal antibodies to purified *Torpedo* AChR were coupled to Sepharose 4B by the method described in

section 3.2.2. The protein was coupled at 1.5mg/ml gel. More than 90% of the protein was bound.

3.3.4 Purification of Torpedo Acetylcholine Receptor on an Anti-(Torpedo AChR) Monoclonal Antibody Affinity Column

Purification by this method employed a modification of that of Lennon *et al* (1980). The frozen tissue was homogenised in 0.4M NaCl and then centrifuged at 100,000g as described above (method 3.3.1). The pellet was then resuspended and the receptor solubilised in 10mM potassium phosphate buffer, pH 7.5, containing 100mM NaCl and 1% sodium cholate for 2h at 20°C, and then centrifuged at 100,000g for 1h. The supernatant was then mixed with anti - (*Torpedo* AChR) monoclonal antibodies, covalently linked to Sepharose 4B, for 2h at 20°C. The gel was washed in a column with 20mM potassium phosphate buffer, pH 7.5, containing 0.2% sodium cholate (overnight at 4°C, 500ml). The receptor was eluted from the column with 0.2M glycine/NaOH, 500mM NaCl, 1% sodium cholate, pH 10.0. The protein peak (monitored at 280nm) was immediately neutralised with 10mM potassium phosphate, 0.2% sodium cholate, pH 7.5, and dialysed extensively against the same buffer. The dialysate was concentrated on a DEAE cellulose column as described above (section 3.3.1).

3.4 SUGAR AND SIALIC ACID DETERMINATIONS

3.4.1 Sialic Acid Determinations

Sialic acid in column eluates and purified AChR was determined by hydrolysing a sample (100µl) with 0.2N H_2SO_4 at 80°C for 1h followed by assay of the free sialic acid using thiobarbituric acid as described by Warren (1959) and modified by Aminoff (1961). After hydrolysis, distilled water (50µl) was added followed by 25mM sodium periodate in 0.125N H_2SO_4 (125µl) and the mixture was incubated for 30min at 37°C. 2% (w/v) sodium arsenite in 0.5M HCl (100µ) was then added followed by thorough mixing and the addition of 0.1M thiobarbituric acid (1ml). The mixture was boiled for 5 - 7 min and the colour was extracted by the addition of butan-1-ol containing 5% (v/v) conc. HCl. The optical density (OD) of the extracted colour was measured at 550nm. Quantitative estimation of the sialic acid was carried out by reference to a calibration curve based on N-acetylneuraminic acid.

3.4.2 Hexose Determinations

The presence of hexose in column fractions was determined colorimetrically by a modification of the cysteine/sulphuric acid assay (Dische and Danilchenko,

1967). Samples (100µl) of column fractions were incubated with a solution containing 0.06% (w/v) cysteine hydrochloride in 60% sulphuric acid (600µl) at 100°C for 3 min in acid washed glass tubes. The samples were then cooled and further 60% H_2SO_4 (600µl) was added and vigorously mixed. The OD of the resulting solutions were read at 420nm.

3.4.3 Acid Hydrolysis and Alditol Acetate Derivatisation of Acetylcholine Receptor Monosaccharides

Individual sugars, including amino-sugars, were determined in the purified AChR by gas-liquid chromatography. Quantitative determinations were done by using alditol acetate derivatives, comparing the detector responses for individual sugars with those of identically treated standards. Derivatisation is a three step procedure involving acid hydrolysis (to release bound monosaccharides), reduction by alkaline borohydride treatment (to form alditols) and finally peracetylation by treatment with acetic anhydride.

Samples (approx. 0.05 - 1.0µg) of purified AChR were evaporated to dryness in 1ml microflux vials in a gentle stream of N_2 at 50°C. 2N Trifluoroacetic acid (TFA) (50µl), containing perseitol (10-25µg) as an internal standard, was added to each sample and the vials were sealed and incubated at 120°C for 1h after which time they were dried as described above. 2M ammonium

hydroxide (100µl) was added to ensure alkaline conditions. The reduction of the free monosaccharide was then carried out by the addition of freshly made sodium borohydride (25µl, 20mg/ml) in 3M NH_4OH. The sealed vials were incubated for 1h at 20°C. Excess borohydride was neutralised by the addition of glacial acetic acid (1-3 drops) followed by addition of methanol (200µl) and dried as above. The residue was washed twice with methanol, twice with methanol-water (1:1) and twice with dry methanol. The residue was further dried by standing over phosphorous pentoxide overnight in a dessicator. Acetylation was carried out by incubation with acetic anhydride (50µl) at 120°C for 1h. The samples were dried and the residue taken up in water (50µl). The alditol acetates were extracted from water with chloroform. The chloroform extract was then dried and the residue dissolved in dichloromethane (10-100µl).

Standards were prepared by derivatisation of equimolar mixtures (50µl) of standard hexoses (L-fucose, D-mannose, D-glucose and D-galactose), hexosamines (N-acetyl-D-glucosamine, and N-acetyl-D-galactosamine) and the internal standard (Perseitol) under the same conditions as described for AChR.

3.4.4 Gas-liquid Chromatography

Gas-liquid chromatography was performed by injection of samples (0.5 - 1.0µl) of the dichloromethane

solution directly into columns (2m x 0.25cm) of coiled glass containing 3% OV 225 on High Performance Gas Chrom Q in a Perkin-Elmer Sigma 3 (OV 225) gas chromatograph, equipped with a dual flame ionization detector. Chromatography was carried out isothermally at 210^{o}C with a carrier gas (nitrogen) flow rate of 45ml/h. Determination of the quantity of individual sugars present in individual samples was achieved by reference to the internal standard by using the following formula

Wt. of monosaccharide =

$$\frac{\text{Peak area of monosaccharide}}{\text{Peak area of perseitol}} \times KF \times \text{Wt. Perseitol}$$

where KF = $\dfrac{\text{Peak area of Perseitol}}{\text{Peak area of equal Wt. of monosaccharide}}$

Individual monosaccharides were identified by comparison of their retention times with those of the standards.

3.5 ENZYME AND ALKALINE BOROHYDRIDE TREATMENT OF ACETYLCHOLINE RECEPTOR

In some instances, samples of purified AChR (in 10mM potassium phosphate buffer, 0.1% Triton X100 (v/v), pH 7.4) were concentrated on a Minicon concentrator prior to glycosidase treatment. The pH of the AChR solutions

was adjusted to the optimum (or mean optimum pH for mixtures) for each enzyme by the addition of 100mM citric acid containing 1mM PMSF and 1mM benzethonium chloride (to prevent proteolysis). All enzyme treated AChR samples were subjected to column chromatography (unless otherwise stated) to separate the protein from cleaved sialic acid or sugars (Sephadex G25, see section 3.7.1) or enzymes (ACA 34, see section 3.7.2).

3.5.1 Neuraminidase Treatment of Torpedo Acetylcholine Receptor

Samples of purified (concentrated) AChR (5 - 10mg in 1.5 - 3.0ml buffer, pH 5.8) were incubated with a solution of neuraminidase (30mU in sodium acetate buffer, 0.05mM, pH 5.5, containing 154mM NaCl, and 9mM $CaCl_2$) at $37^{o}C$ for 2 - 3h.

3.5.2 Mixed Endoglycosidase D Treatment

Mixed endoglycosidase D consisted of neuraminidase, β-galactosidae, β-N-acetylglucosaminidase and

endoglycosidase D (1 U/ml, 2.3 U/ml, 13.0 U/ml and 5 U/ml respectively). AChR in phosphate buffer (pH 6.5) was incubated with a solution of the mixed endoglycosidases (10mU in 5mM potassium phosphate buffer, pH 6.5) for 6h at 37°C.

3.5. T.foetus Enzyme Treatment

Crude preparations of T.foetus enzymes (9mg samples) were further purified on a column of Sephadex G25 (1.2cm x52cm). The column was eluted under gravity with 10mM potassium phosphate buffer, pH 6.0, containing 1% BSA. Fractions of 1ml were collected and the protein peak (as measured at 280nm) was pooled and stored at 4°C in 100mM potassium phosphate buffer, pH 6.0, containing 1mg/ml BSA.

The pH of the Torpedo receptor preparations (1.3mg) was adjusted to pH 6.0 prior to incubation with T.foetus enzymes (3mg). Incubation was carried out for 5h at 37°C

3.5. Alkaline Borohydride Treatment

Purified lyophilised AChR (6.5mg) was treated with 0.05M sodium hydroxide containing 1M potassium borohydride (1ml) at 50°C for 20h. The solution was then neutralised by the dropwise addition of glacial acetic acid prior to column chromatography on Sephadex G25 (section 3.7.1).

3.6 ASSAYS

3.6.1 Assay for Glycosidase Activity

Assay of β-galactosidase activity was carried out by a modification of the method of Distler and Jourdian (1973). A sample (100µl) of solution was added to 10mM potassium phosphate buffer, pH 6.4, (900µl) containing 5mg/ml O-nitrophenyl-β-D-galactopyranoside and 0.2% BSA. The mixture was incubated for 30 min at 37°C after which 0.25M glycine, pH 10.0, (1ml) was added and the absorbance read at 420nm.

3.6.2 Protein Assay

Total protein in aqueous solutions was determined colorimetrically by the method of Lowry *et al* (1959). 5% sodium dodecyl sulphate (SDS) was included in the alkali buffer for samples containing Triton X100 (Dulley and Grieve, 1975)

3.7 GEL FILTRATION OF TORPEDO ACETYLCHOLINE RECEPTOR

All column chromatography was performed at room temperature. Gels were swollen and columns packed according to the manufacturers' instructions. Gels and elution buffers were degassed before use and columns were

pre-equilibriated in elution buffers. Flow rates were maintained with a peristaltic pump and samples automatically collected with an LKB Redirak 2112 fraction collector with an electronic timer. Columns were monitored continuously (at 280nm) with a Cecil 272 Spectrophotometer fitted with a flow cell (500µl volume) and coupled to a Bryans 28000 chart recorder.

3.7.1 Sephadex G25 (medium) Chromatography

Small molecular weight molecules were separated from Torpedo acetylcholine receptor (approx. weight 250,000) by gel filtration on Sephadex. The column (1.8cm x 82.5cm) was eluted with 10mM ammonium acetate buffer, pH 7.4, containing 0.01% thiomersol (to prevent bacterial contamination). Elution was carried out at a flow rate of 30ml/h. Fractions of 3ml were collected in glass tubes.

3.7.2 Ultragel ACA 34 Chromatography

Gel filtration of some treated and untreated samples of acetylcholine receptor (approx. weight 250,000) was carried out on a column (1.2cm x 51cm) of Ultragel ACA 34 (exclusion limit of 35000 for globular proteins). The column was eluted at 12ml/h with 10mM potassium phosphate buffer, pH 7.4, containing 0.3M NaCl and 0.02% sodium azide. Fractions of 1ml were collected.

The column was calibrated by using Dextran blue (M.Wt 2 x 10^6), IgG (M.Wt 150,000), Ovalbumin (M.Wt 43,000), Myoglobin (M.Wt 18,000) and DNP lysine (see Figure 3.7.1).

3.7.3 Sephacryl S200 (Superfine) Chromatography

Gel filtration of some untreated samples of acetylcholine receptor (approx. 1.5 - 2.5mg) was carried out on a column (1.5cm x 30cm) of Sephacryl S200. The column was eluted at 60ml/h with 10mM potassium phosphate buffer, pH 7.4, containing 0.3M NaCl and 0.02% sodium azide. Fractions (1ml) were collected. The column was calibrated as for the ACA 34 column (see Figure 3.7.2).

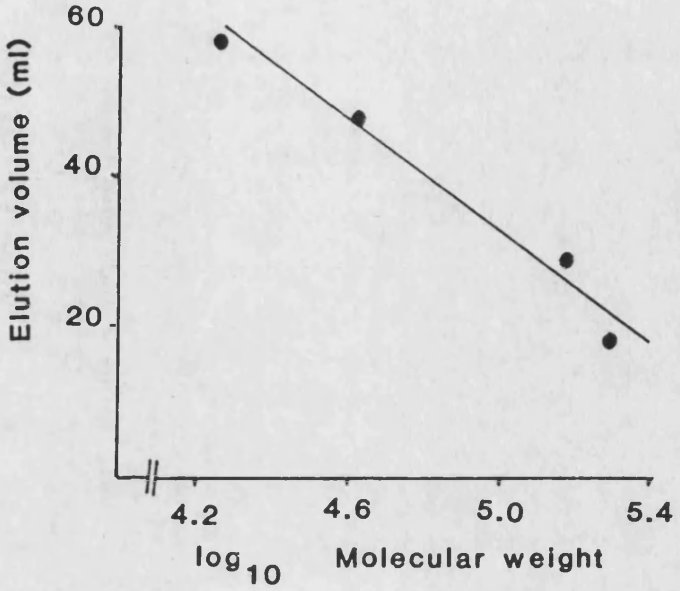


Figure 3.7.1 Calibration plot of standard proteins on Ultragel ACA 34.

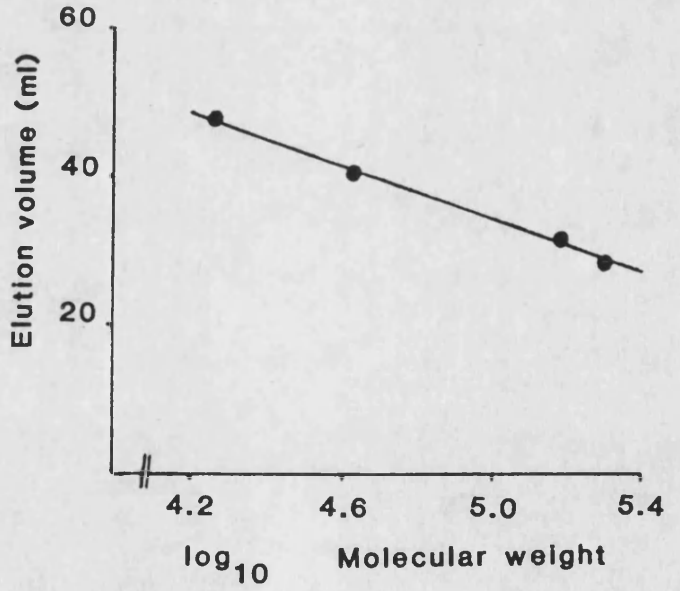


Figure 3.7.2 Calibration plot of standard proteins on Sephacryl S200.

3.8 GEL ELECTROPHORESIS AND ISOELECTRIC FOCUSSING

3.8.1 Isoelectric Focussing

Isoelectric focussing was carried out in tubes 0.4cm x 20cm using gels consisting of 5% acrylamide, 1% Triton X100, 7% ampholines (4.37% ampholine pH 5 - 7, 2.63% ampholine pH 3.5 - 10), 0.2% (v/v) TEMED, 0.3% (w/v) ammonium persulphate. The gels were overlayed with water and allowed to set. Production of pH gradients in the gels was achieved by pre-running for 1 or 2h at 400V or 200V respectively. Samples of purified AChR (with or without enzyme treatment) were incubated with ^{125}I - αBGT (± benzoquinonium chloride) for 90 min at 20°C prior to being loaded onto the gels. Samples of the AChR/^{125}I - αBGT mixtures after incubation were loaded onto the gels in glycerol and subjected to isoelectric focussing for 5h at 1000V or overnight at 500V. Buffers used were:-

Cathode, 0.4% (v/v) H_2SO_4, 1% Triton X100.

Anode, 0.01N NaOH, 1% Triton X100.

After focussing the gels were either sliced immediately or first frozen at -80°C. Slices of 2mm or 5mm were put in a disposible tube with 0.4ml water and mixed. Radioactivity was counted in a gamma counter and the pH of each fraction was measured after 2h.

3.8.2 SDS - Polyacrylamide Gel Electrophoresis (SDS PAGE)

i) Disc and slab gels were made and run according to the method of Laemmli (1970) using 7.5% or 10% acrylamide.

ii) 5 - 14% gradient acrylamide slab gels were poured and run as follows:-

Gels of 5 - 14% acrylamide (0.35% N,N,methyl bisacrylamide) in 0.375M Tris HCl pH 8.9 containing 15% (w/v) sucrose, 0.5 M urea, 0.1% (w/v) SDS and 0.01% (w/v) ammonium persulphate were poured by using a glass gradient mixer and a peristaltic pump (to maintain a constant flow). The gels were overlayed with water and allowed to set. Once set, the water was poured off and a stacking gel of 4% acrylamide in 0.125M Tris HCl, pH 6.6 (containing 18% sucrose, 0.5M urea, 0.11% SDS and 0.5% ammonium persulphate) was overlayed on the gradient gel. Wells were preformed in this gel by using a plastic comb.

The gels were allowed to set overnight at room temperature prior to running.

Samples were run into the gel at 10mA, allowed to run through the stacking gel at 15mA and through the running gel at 30mA. The electrophoresis buffer consisted of 0.025M Tris containing 0.05M glycine, 0.1% SDS, 0.5M urea.

Samples of AChR and standard proteins (10 - 200µl)

were denatured by incubating in β-mercaptoethanol and SDS at 100°C for 2 - 10 min. Standards routinely used for gel electrophoresis are shown in Table 3.8.1. Bromophenol blue was routinely used as a tracking dye. Electrophoresis was carried out until the tracking dye was within 4cm of the end of the gel.

3.8.3 Coomassie Blue Staining of Gels

Gels were either stained for 2 - 12h in a solution of Coomassie brilliant blue R 250 (1.25g/500ml) in 45% (v/v) methanol, 10% acetic acid, or in a solution of 0.04% (w/v) Coomassie brilliant blue R (250) in 12.5% (w/v) tricloroacetic acid (TCA) for 6 - 24h. Destaining was carried out in 30% methanol, 10% acetic acid (method 1) or in 12.5% TCA (method 2).

3.8.4 Visualisation of Carbohydrates in SDS Polyacrylamide Gels

SDS polyacrylamide gels were stained with Schiffs reagent according to Segrest and Jackson (1972). The Schiffs reagent was prepared as follows:- basic fuschin (2.5g) was dissolved in 500ml boiling water and then cooled to 60 - 80°C. Sodium metabisulphite (5g) and 1M HCl (50ml) were added and stirred until the solution was decolourised. After cooling to 4°C, HCl-washed charcoal (1.5g) was added and the solution stirred for 1 min. The

Table 3.8.1 Molecular Weights of Standard Proteins used in Gel Electrophoresis

PROTEIN	MOLECULAR WEIGHT
Glucose oxidase	80,000
Mucin	74,000
IgM (heavy chain)	74,000
(light chain)	25,000
BSA	63,000
IgG (heavy chain)	50,000
(light chain)	25,000
Ovalbumin	42,000
Casein	18,000
Myoglobin	17,200
Ribonuclease	13,700
Cytochrome c	11,700

Schiffs reagent was rapidly filtered through filter paper and stored in a brown bottle at $4^{o}C$.

The gels were stained for glycoproteins by the following method. After electrophoresis, gels were fixed in 40% methanol, 5% acetic acid at room temperature for 24h. The gels were then placed in a solution of 0.7% (w/v) periodic acid in 5% acetic acid for 2 - 3h (in the dark) with one change. Free iodine was removed from the gel by repeated washing in 0.2% sodium metabisulphite, 5% acetic acid until all the brown colour was removed. The gel was then placed in the Schiffs reagent. Colour developed in up to 2h. After colour development, the gel was repeatedly washed in 0.1% sodium metabisulphite, 0.01N HCl. The gels were then photographed.

3.8.5 Staining of Glycoproteins in Polyacrylamide Gels with FITC-Labelled Lectins

Staining for carbohydrates with fluorescein isothiocyanate (FITC) labelled lectins was carried out according to Furlan *et al* (1979).

SDS polyacrylamide slab gels were fixed overnight in methanol:acetic acid:water (10:3:27) and then equilibriated in 0.1M NaCl , 50mM Tris HCl (pH 7.0) containing 1mM chloride salts (CA^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}). FITC - lectins, solubilised in the same buffer (50µg/ml) were overlayed on the gels and left at $20^{o}C$ for 20 - 72h. Destaining was carried out in buffer alone. The gels

were then photographed.

3.9 DETECTION OF CARBOHYDRATE RESIDUES AND STAINING OF GLYCOPROTEINS IMMOBILISED ON NITROCELLULOSE PAPER

3.9.1 Electrotransfer

Proteins from SDS-PAGE were transferred onto nitrocellulose paper according to a modified method of Bittner *et al* (1980). When gels were run for this purpose 0.2% methyl green was used as the tracking dye. 30 min before the end of electrophoresis a further amount (1µl) of methyl green was added to each well and allowed to run into the running gel. Methyl green remains bound to the nitrocellulose allowing channels to be accurately located.

After electrophoresis, gels were briefly rinsed in water and then layered onto a sheet of pre-wetted (in water) nitrocellulose paper and sandwiched between pre-wetted Whatman filter paper (3mm). Precautions were taken to ensure that no air bubbles were trapped between the gel and nitrocellulose paper. The sandwich was then placed between Scotchbrite pads and supported between a nylon grid. Transfer was carried out in 30mM sodium phosphate buffer, pH 6.6, overnight with a constant current of 0.2A. The buffer was stirred to avoid local heating effects.

The nitrocellulose was either immediately stained

for protein or was quenched in 50mM Tris-saline, pH 7.4, containing 0.25% (w/v) gelatin, 0.05% (v/v) Tween, 0.01% (w/v) thiomersol, 1mM salts (Ca^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}) (blocking buffer) for 2h at 37°C or overnight at 20°C.

3.9.2 Amido Black Staining of Proteins

After electrotransfer, sheets of nitrocellulose were washed in water for 10 min and then stained for 10 - 15 min in 0.1% amido black in 45% methanol, 10% acetic acid. Destaining was carried out in 50% methanol, 10% acetic acid. The nitrocellulose was washed in water, dried and stored between sheets of filter paper in the dark.

3.9.3 Preparation of Glycoprotein-Horseradish Peroxidase Conjugate

Glycoproteins were conjugated to horseradish peroxidase (HRO) by using a modified method of Wilson and Nakane (1978) for conjugating antibodies to HRO.

Horseradish peroxidase, dissolved in water (2ml), was oxidised by stirring with 0.1M sodium periodate (400µl) in the dark at 20°C for 30 min. Periodate was removed from the solution by dialysing against 1mM sodium acetate buffer, pH 4.4, for 1h with 2 changes, and then overnight at 4°C. The pH of the dialysate was then raised to 9.5 with 0.2M sodium carbonate/bicarbonate

buffer (40µl). Molar equivalents of the protein to be conjugated (in 10mM sodium carbonate/bicarbonate buffer, 1ml) were immediately added and stirred for 4h at 20°C. The conjugate was then reduced by the addition of freshly prepared sodium borohydride (5mg/ml in water, 100µl). The mixture was allowed to stand at 4°C for 2h (a second sample of $NaBH_4$ (100µl) was added after 1 h) and then dialysed against phosphate buffered saline (PBS). Samples of the conjugates not required for immediate use were stored at 4°C.

In some instances the protein-HRO conjugate was separated from free HRO by gel filtration on Sephadex G75 (1.5cm x 100cm). Fractions (2.5ml) were collected and the column was eluted with PBS at a flow rate of 20ml/h. Fractionated and unfractionated HRO-conjugates were stored in glycerol (1:1, v/v).

Approximation of HRO-conjugate concentration

HRO concentration (mg/ml) = A403/1.5

Protein concentration (mg/ml) =

$$\frac{A280 - (A403/1.5)}{\text{Extinction coefficient of the protein}}$$

$$\text{Molar ratio} = \frac{\text{HRO conc.}}{\text{Protein conc.}} \times \frac{\text{Mr Protein}}{\text{Mr HRO}}$$

3.9.4 Spot Tests

i) For testing glycoprotein-HRO conjugates

Lectin solutions (1µg) were spotted onto nitrocellulose paper (pre-wetted for 30 min and dried for 5 min between filter paper) and allowed to dry for 30 min at 37°C. The sheets were then incubated with blocking buffer for 2h at 37°C and incubated with the glycoprotein-HRO conjugate overnight at 20°C after which time they were washed (x 3) with blocking buffer for 30 min (with shaking). A final wash (for 10 min) was carried out in 50mM sodium acetate buffer, pH 5.0. Colour was developed by incubation with 0.02% 3-amino-9-ethyl carbazole (dissolved in dimethyl-formamide, 0.4% solution (w/v))., 0.03% hydrogen peroxide in 50mM sodium acetate, pH 5.0. After development of colour, the sheets were rinsed in water, dried and either photographed or stored between sheets of filter paper in the dark.

ii) For the detection of carbohydrate residues in AChR

AChR (1 - 5µg) was spotted onto nitrocellulose paper, dried and blocked as in i). The sheets were incubated with solutions (10 - 20µg/ml) of lectins in blocking buffer for 4h at 37°C (with shaking) or overnight at 20°C. They were then washed (x 3) for 30 min with blocking buffer and incubated with a suitable dilution of the HRO-conjugate. Further procedures were then carried out as in i).

3.9.5 Detection of Carbohydrate residues in Glycoproteins from Protein Blots

Glycoproteins electrophoretically transfered from gels onto nitrocellulose (section 3.9.1) were quenched and treated as in ii) above.

3.10 ENZYME-LINKED LECTIN BINDING ASSAY (ELBA)

Lectin binding to glycoproteins and hence the detection of carbohydrate residues was demonstrated by a modification of the enzyme - linked immunosorbant assay (ELISA).

250µl plastic cuvettes were coated with a solution (5µg/ml) of glycoprotein in 50mM sodium bicarbonate, pH 9.6, overnight at 4°C. The cuvettes were washed briefly

with PBS, incubated with 1% gelatin in PBS for 30 min at 20°C to block non-specific binding sites and then washed 3 x 30 min in PBS containing 0.05% (v/v) Tween 20. The cuvettes were then incubated with various concentrations of Concanavalin A (Con A) or HPO-Lotus tetragonolobus, in PBS for 2h at 37°C or overnight at 20°C. Washing was carried out with PBS - Tween as before. A solution of HRO (1µg/ml, 250µl) in PBS was incubated as for the lectins (for the cuvettes incubated with Con A) and washed as before. Colour was developed by adding 0.04% (w/v) o-phenylenediamine in phosphate - citrate buffer, pH 5.0, containing 0.012% (v/v) hydrogen peroxide to the samples and incubation at RT. After development of colour the reaction was stopped by the addition of 60% H_2SO_4 (50µl) and the colour read (after mixing) at 490nm.

3.11 HAEMAGGLUTINATION TESTS

3.11.1 Neuraminidase Treatment of Human Red Blood Cells (Group A)

Human red blood cells (group A) were washed (5 times) with PBS by centrifugation at 300g for 10 min. They were resuspended (10% v/v) in PBS, pH 7.4, containing 1mM $CaCl_2$ and neuraminidase (50µl, 1 IU/ml) and the mixture was incubated at 37°C for 30 min. The treated cells were washed twice as before and resuspended

in PBS at a concentration of 2% (v/v).

3.11.2 Haemagglutination Activity of Lectins

The activity of lectin solutions was checked by the haemagglutination test. Lectin solutions (1mg/ml, 25µl) were serially diluted in Tris - saline, pH 7.2, (25µl, containing salts as in section 3.9.1) in U-bottomed microtitre trays. To each well was then added a 2% (v/v) suspension of human red blood cells (25µl) in PBS. The end points were determined after 2h incubation at 20°C by the settled pattern technique (Ling et al., 1977). The lectins tested are shown in Table 3.10.1. With the exception of Limulus polyphemus lectin, all lectins showed strong binding to red blood cells (Table 3.10.2).

3.11.3 Inhibition of Lectin-Induced Haemagglutination by Torpedo Acetylcholine Receptor

Torpedo acetylcholine receptor (270pmol, 25µl) was serially diluted with Tris-saline, pH 7.2, in U-bottomed microtitre plates. Samples of various lectins (40µg, 25µl) were preincubated with the receptor for 15 min at room temperature after which time a 2% (v/v) suspension of human red blood cells (25µl) (type O or A) was added in PBS. The end points were determined, by the settled pattern technique, after 2h at room temperature. Controls were red blood cells only and serial dilution of

Table 3.11.1 Blood Group Used to Test the Activity of Various Lectins

BLOOD GROUP	LECTIN
A	Lens culinaris
	Triticum vulgaris
	Succinylated T.vulgaris
	Pisum sativum
	Glycine max
Neuraminadase treated A	Arachis hypogaea
	FITC labelled A.hypogaea
O	Canavalia ensiformis (Con A)
	Limulus polyphemus
	Lotus tetragonolobus
	HPO labelled L.tetragonolobus
	Ulex europeus

Table 3.11.2 Activity of Various Lectins to RBC

LECTIN	END POINT (μg/ml x 10^{-3})
L.culinaris	7.8
T.vulgaris	7.8
Succinylated T.vulgaris	3.9
P.sativum	15.6
G.max	7.8
A.hypogaea	>0.9
FITC labelled A.hypogaea	15.6
C.ensiformis (Con A)	1.2
L.polyphemus	>500
L.tetragonolobus	15.6
HPO-labelled L.tetragonolobus	3.9
U.europeus	62.5

Triton X100 buffer (10mM potassium phosphate, pH 7.4, 0.1% Triton X100, 0.1% azide) in Tris-saline, pH 7.2 followed by incubation with red blood cells.

3.12 PASSIVE HAEMAGGLUTINATION TEST

3.12.1 Attachment of Acetylcholine Receptor to Sheep Red Blood Cells (SRBC)

Fresh sheep red blood cells (SRBC) were coated with AChR by the chromic chloride procedure of Ling et al (1977). The receptor was dialysed against saline (0.14M NaCl) for 24h prior to coating. 0.5ml of a 10% (v/v) suspension of red cells was washed five times in saline by centrifugation (400 x g, 5 min) and the supernatant discarded after the final wash. To the cell pellet was added 0.30ml of AChR solution (0.15mg) and, with vortex mixing, the required amount of 0.1 mg/ml aged chromic chloride ($CrCl_3.6H_2O$) in saline solution (0.10 - 0.40ml) was added. Vortexing was continued for a further 10 seconds, then saline (2ml) was layered over the cells and the preparation left at 4°C overnight. The cells were then washed three times with Hepes-buffered Eagles medium (containing 100µg/ml streptomycin and 100 units/ml penicillin) and finally suspended at 1% (v/v) in H-Eagle containing, 5% (v/v) heat-inactivated foetal calf serum (FCS), and stored at 4°C. Red cells coated with bovine haemoglobin (Hb) by the above procedure were used as

controls.

3.12.2 Passive haemagglutination and complement - mediated lysis assays

Serial dilutions of antiserum were performed in 0.05ml volumes of H-Eagles-FCS in microtitre trays. 0.025ml of 1% coated cells was added and the end-point read by the settled pattern technique after 2 - 4h incubation at 20°C.

Complement-mediated lysis was set up as for the agglutination test with the following modifications: phosphate buffered saline, pH 7.3, containing 1% bovine serum albumin (BSA/PBS) was used as the diluent and, prior to the addition of coated red cells, 0.025ml of guinea pig complement (diluted 1:4 in BSA/PBS) was added. The microtitre plates were incubated in a humid chamber for 2h at 37°C and the end-point (last well showing lysis) was recorded.

3.12.3 αBungarotoxin binding to AChR-coated red cells

Increasing amounts of ^{125}I-αBGT (0.034 - 4.77 pmole) were added to 0.020ml aliquots of a 1% (v/v) suspension of coated red cells. The volume was made up to 0.2ml with H-Eagle-FCS and the mixture incubated for 90mins at room temperature with occasional mixing. The cells were then washed three times (400 x g, 10 min) with

H-Eagle-FCS medium (0.5ml) and bound radioactivity was counted in an LKB 1280 Ultragamma Counter. Specific binding (70 - 80% of total) was defined as binding of ^{125}I-αBGT that is displaceable by 63mM benzoquinonium chloride.

4. RESULTS

4.1 BUNGAROTOXIN STUDIES

Various studies were carried out on iodinated αBGT in order to assess the accuracy of the Toxin Binding Assay for the acetylcholine receptor. Competition studies were carried out to verify values (obtained by gel filtration) for the concentration of iodinated αBGT. The αBGT iodinated by the standard Chloramine T method was compared with toxin iodinated by other methods.

4.1.1 Purification of the α-Toxin from Bungarus multicinctus Venom

In order to investigate the purity of commercially available αBGT the α-toxin was isolated from Bungarus multicinctus venom. Figure 4.1.1 shows the elution profile of B.multicinctus venom on a column of CM Sephadex. Peak 2 from this column was rechromatographed on a column of CM cellulose (Figure 4.1.2).

Peak 1 from the CM Sephadex column is reported to be guanosine, peaks 2 and 3 are α-toxins and further peaks β-toxins (Lee et al., 1972). Rechromatography of peak 2 on CM cellulose resolves it into two peaks, both α-type neurotoxins. Fraction 2_2 (α-bungarotoxin) is reported to be the most potent and is free of acetylcholinesterase activity (concentrated in a narrow

FIGURE 4.1.1 Chromatography of Bungarus multicinctus venom (50mg) on a CM-Sephadex column (2.0cm x 25cm) by gradient elution with ammonium acetate buffer (340ml) of 0.05M (pH 5.0) - 0.5M (pH 7.0) (gradient represented by dotted line). Eluates of 3ml were collected with a flow-rate of 15ml/h.

FIGURE 4.1.2 Rechromatography of peak 2 (from CM-Sephadex column, see figure 4.1.1) on a CM-cellulose column (0.8cm x 14cm) by gradient elution with ammonium acetate buffer (200ml) from 0.05M (pH 5.0) - 0.2M (pH 7.0) (gradient represented by dotted line). Eluates of 1.4ml were collected with a flow-rate of 9.3ml/h.

FIGURE 4.1.1

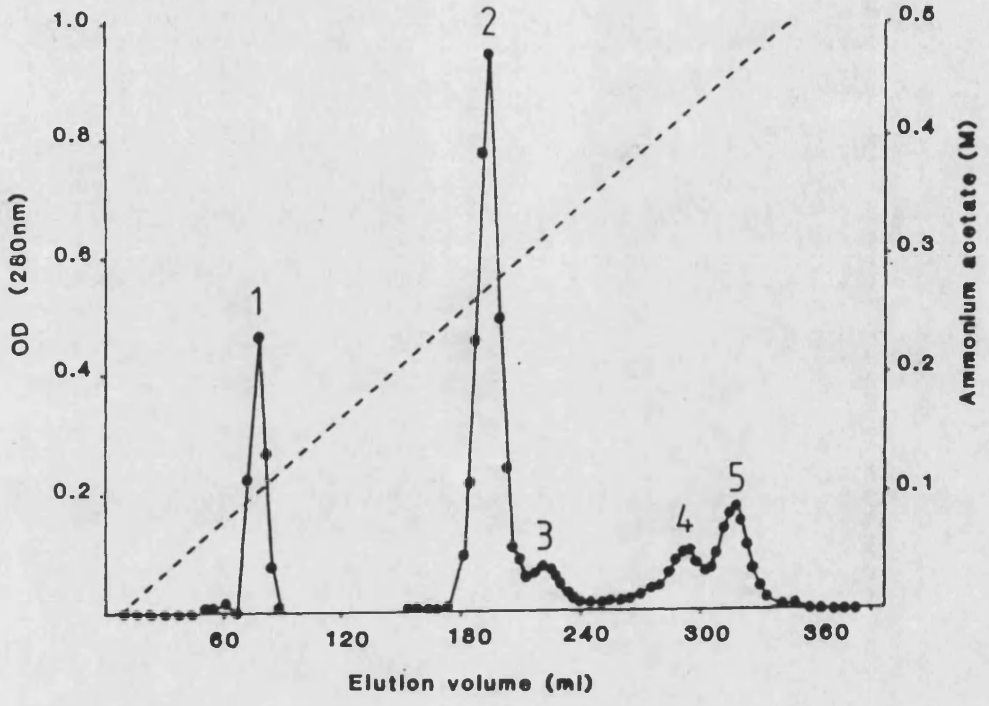
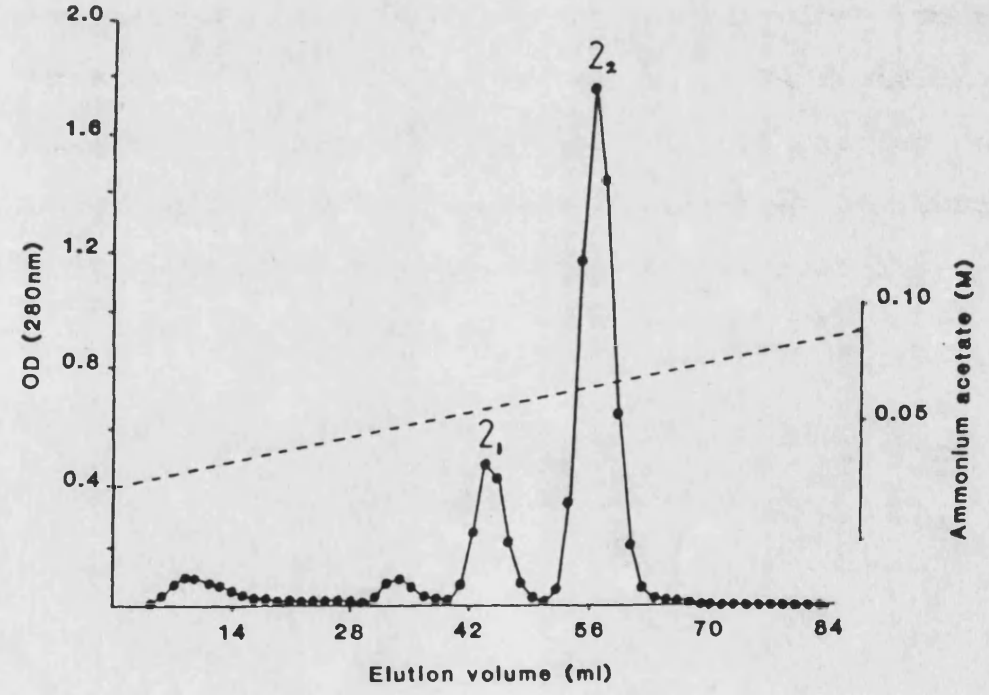


FIGURE 4.1.2



peak around the starting point of fraction 2_1 (Lee et al., 1972). Fraction 2_2 was lyophilised and used in further studies. It comprises approximately 25% (w/w) of the initial protein.

4.1.2 Purity Check of Commercial α-Bungarotoxin

Ion-exchange chromatography of commercial α-bungarotoxin was carried out on CM Cellulose. The elution profile of the αBGT from the CM cellulose column is shown in Figure 4.1.3. This Figure shows two protein peaks. The major one contained 85% of the protein and it showed α-toxin. The repurified αBGT was subjected to further investigations, see sections 4.1.5 and 4.1.6.

4.1.3 Comparison of Methods of Iodination of α-Bungarotoxin: Comparison of Methods

Commercial α-BGT was iodinated by the Chloramine T method (Hunter, 1978). It was also iodinated by the oxidative iodine monochloride (ICl) method (Vogel et al.,1972) and by a modification of the ICl method (Doran and Spar, 1980) in order to compare methods. Specific activities of the ^{125}I - αBGT preparations are shown in Table 4.1.1. The percentage incorporation of ^{125}I into αBGT and specific activity of the preparations using the alternative methods of iodination was always lower than when the Chloramine T method was used. There was no

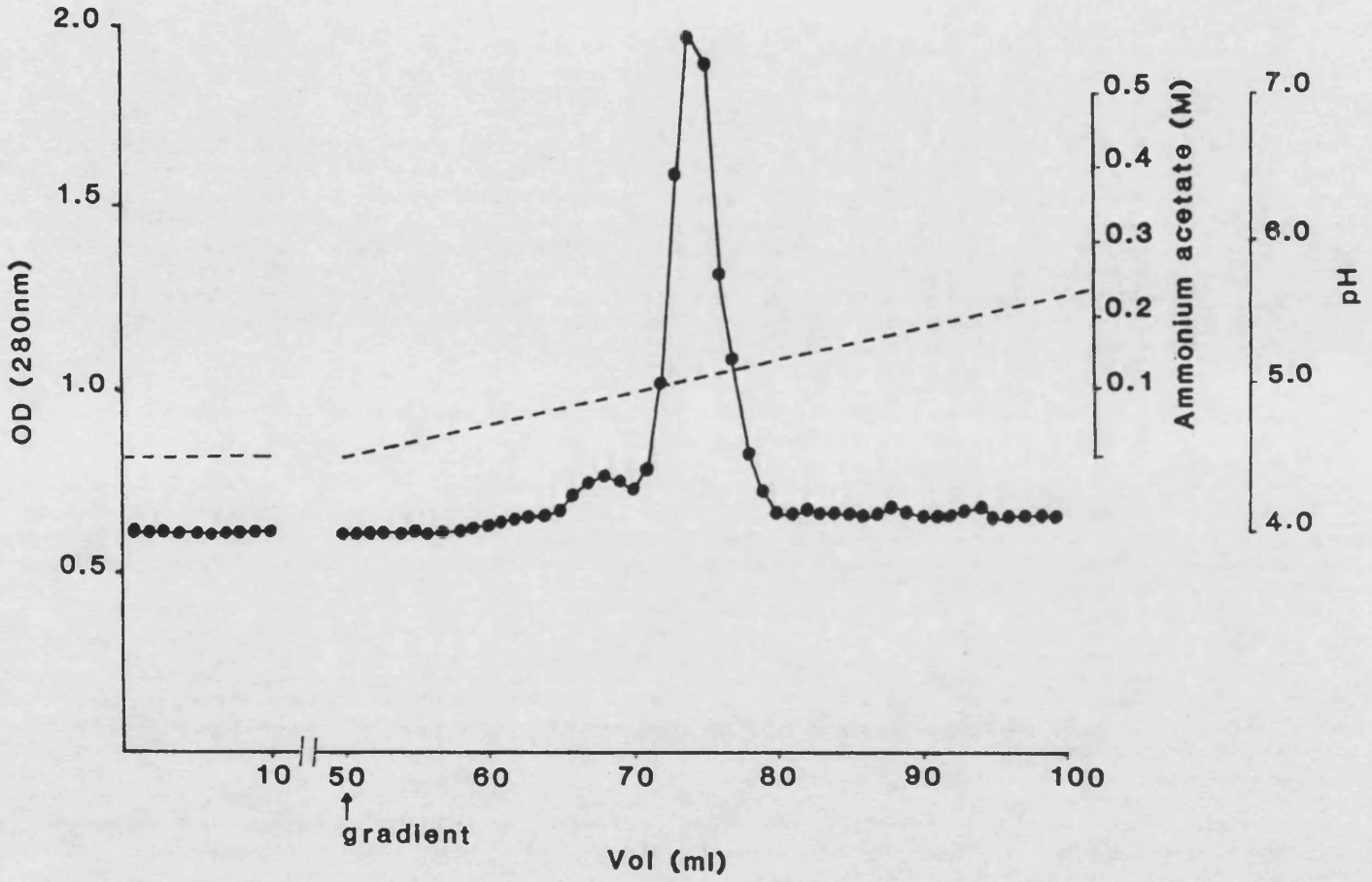


Figure 4.1.3 Chromatography of commercial αBGT (10mg) on CM-cellulose column (0.8cm x 15cm) by gradient elution with ammonium acetate buffer, from 0.05M, pH 5.0 to 0.5M, pH 7.0 (80 ml total). Eluates of 1ml were collected with a flow-rate of 10ml/h.

Table 4.1.1 Specific Activities and Percentage Incorporation of ^{125}I in Various Preparations of αBungarotoxin

METHOD OF IODINATION	SPECIFIC ACTIVITY (Ci/mMol)	PERCENTAGE INCORPORATION of ^{125}I
Chloramine T[1]	763 ± 34	81.2 ± 5 (8)
Oxidative ICl[2]	234	29 (2)
Modified ICl[3]	243 ± 82	23 ± 0.3 (4)

[1] Hunter, 1978

[2] Vogel et al., 1972

[3] Doran and Spar, 1980

± Standard error of the mean

Figures in brackets represent number of experiments

appreciable difference between the values obtained by the IC1 and modified IC1 methods.

4.1.4 Fractionation of Iodinated α-Bungarotoxin

Commercial αBGT iodinated by the Oxidative IC1 method was fractionated on CM Sephadex (see Figure 4.1.4).

The recovery of radioactivity from the column varied between 74% and 81% over 3 preparations. A peak of radioactivity, of between 10 and 34% of the total, was always present in the column wash prior to salt elution.

The concentration of αBGT in the iodinated preparations was determined as described in methods 3.1.12. 20% of the radioactivity applied to the column was recovered as the monoiodinated derivative.

4.1.5 Biological Activity of Radiolabelled α-Bungarotoxin and Specific Binding to AChR

The toxin binding activity of various preparations of iodinated αBGT was investigated by incubating a 1000 fold molar excess of Torpedo acetylcholine receptor with the toxin for 60 min. The percentage of iodinated αBGT bound by the AChR was calculated. Estimations of the biological activity of fractionated and unfractionated toxin are shown in Table 4.1.2. While the biological activity of αBGT iodinated by any method was extremely

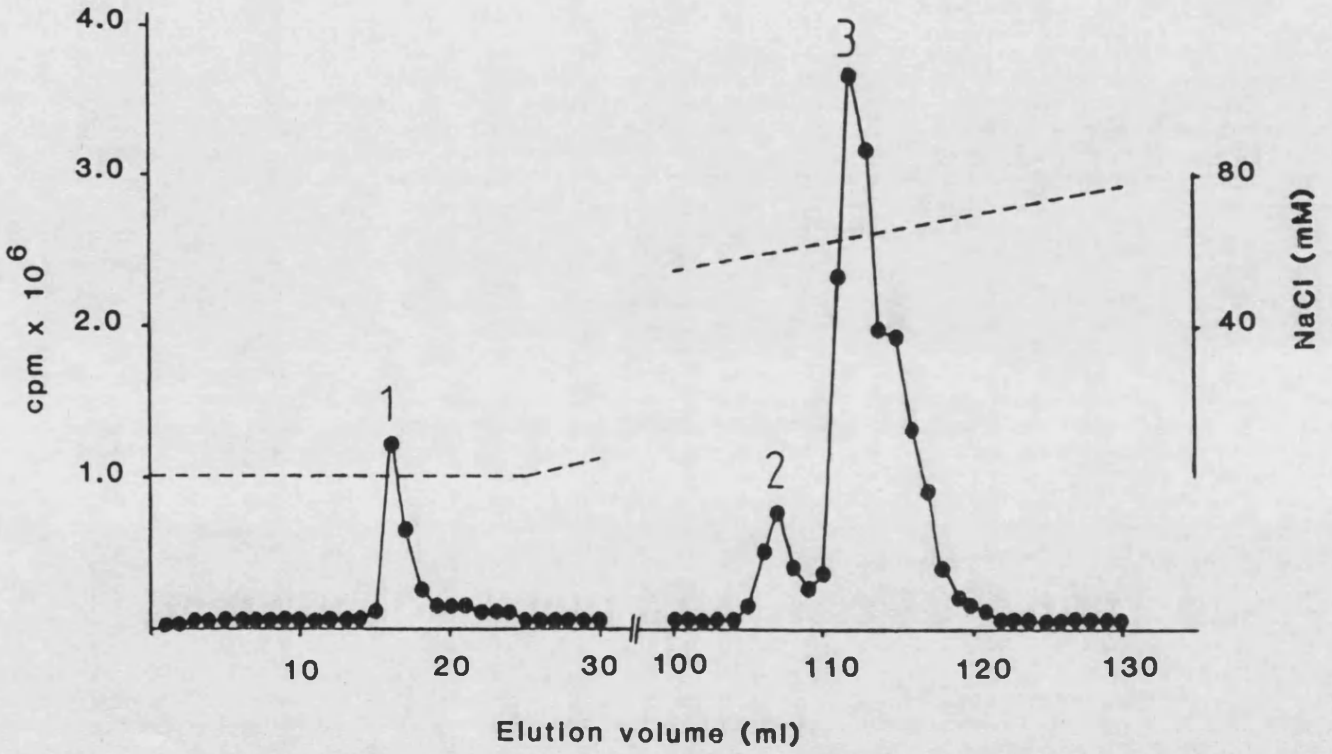


Figure 4.1.4 Chromatography of iodinated αBGT on CM-Sephadex (C50, 1cm x 3cm) by gradient elution with 10mM potassium phosphate buffer, pH7.4, containing 0 - 80mM NaCl (100ml). Fractions of 1ml were collected with a flow-rate of 15ml/h. Samples (10μl) of each fraction were counted for radioactivity. 1. Free [^{125}I]. 2. [$^{125}I_2$] - αBGT. 3. [^{125}I]-αBGT.

Table 4.1.2 Biological Activity of Radiolabelled α-Bungarotoxin

RADIOLABELLED SPECIES	BIOLOGICAL ACTIVITY (%)
Unfractionated ^{125}I αBGT	30 - 75 (17)[a]
	42 - 66 (4)[b]
	13 - 34 (3)[c]
Monoiodinated ^{125}I αBGT	52 - 65 (2)[b]
Diiodinated ^{125}I αBGT	59 - 86 (2)[b]

Method of iodination

a) Chloramine T

b) Oxidative ICl method (Vogel et al., 1972)

c) Modified ICL method (Doran and Spar, 1980)

Figure in brackets indicates number of experiments.

variable, iodination by the modified ICl method always produced αBGT preparations with low biological activities. Estimation of the biological activity of α-BGT from venom and repurified from commercial α-BGT (iodinated by the Chloramine T method) gave values in the range of 35 - 75%. These values were not significantly different from those of the commercial αBGT.

Specific binding of iodinated preparations (unfractionated and fractionated) to Torpedo AChR was determined as described in 3.1.6(i). Results are shown in Table 4.1.3. There was no appreciable difference between the specific binding of fractionated and unfractionated αBGT to Torpedo AChR.

4.1.6 Immunological Activity of Iodinated α-Bungarotoxin

The proportion of radiolabelled repurified commercial αBGT precipitated by anti-(α-bungarotoxin) antibodies was investigated by radioimmunoassay. The immunological activity of commercial toxin and toxin purified from venom was found to be approximately 63% and 68% respectively. Preincubation of antisera with either unlabelled repurified or commercial αBGT or substitution of the immune serum with normal rabbit serum reduced the radioactivity to less than 5% of the total amount added.

Table 4.1.3 Specific Binding of Various Acetylcholine Receptor Preparations to Monoiodinated and Unfractionated α Bungarotoxin

UNFRACTIONATED	MONOIODINATED
1733 ± 167	2098 ± 128
7049 ± 209	7479 ± 603
10186 ± 451	10821 ± 504
5572* ± 380	6410* ± 441

Binding is expressed as pmol AChR bound per ml of receptor preparation

* Separate toxin preparation

± Standard error of mean

4.1.7 Estimation of the Concentration of Iodinated α-Bungarotoxin Preparations: Comparison of Methods

The binding of iodinated commercial αBGT to increasing quantities of Torpedo receptor was plotted for various quantities of unlabelled toxin added. The slopes so derived were plotted against the concentration of unlabelled toxin (see Figure 4.1.5). Values for the concentrations of iodinated α-bungarotoxin were obtained from these slopes by linear regression. Table 4.1.4 compares these results with those obtained by the gel filtration method of quantitation. The two sets of values are not significantly different.

4.1.8 pH Optimum for α-Bungarotoxin Binding to AChR

This investigation was carried out in order to discover the sensitvity of the toxin binding assay to changes in pH. The assay was carried out as described in section 3.1.6. The pH of the toxin binding assay buffer was adjusted to give values in the range of 6.0 - 7.8 at intervals of 0.2. Changes in pH within the range studied had no effect on toxin binding to Torpedo acetylcholine receptor.

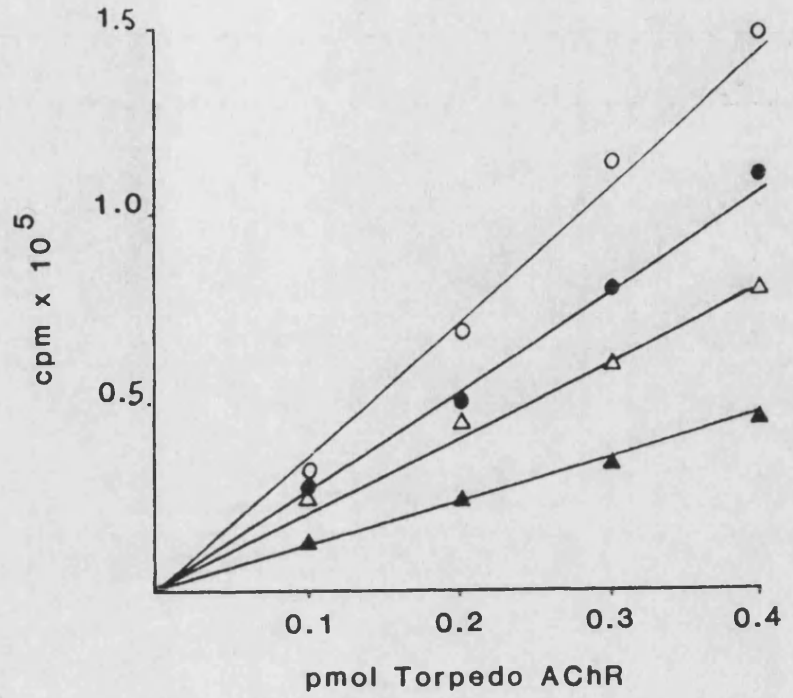
Figure 4.1.5 The quantitation of the concentration of α-bungarotoxin, purified from venom, in a radiolabelled preparation.

a) The binding of iodinated (unfractionated) toxin purified from venom to increasing quantities of Torpedo AChR in the presence of various quantities of unlabelled toxin. (o) 0 pmoles, (●) 2 pmoles, (Δ) 4 pmoles, (▲) 8 pmoles.

b) Slopes derived from (a) plotted against the concentration of unlabelled toxin. The intercept on the abcissa gives the quantity of α-bungarotoxin present in 5μl of the radiolabelled toxin.

A value of 0.70 pmol/μl was obtained by linear regression analysis.

a)


1.5
1.0
0.5
cpm x 10^5
0.1
0.2
0.3
0.4
pmol Torpedo AChR

b)

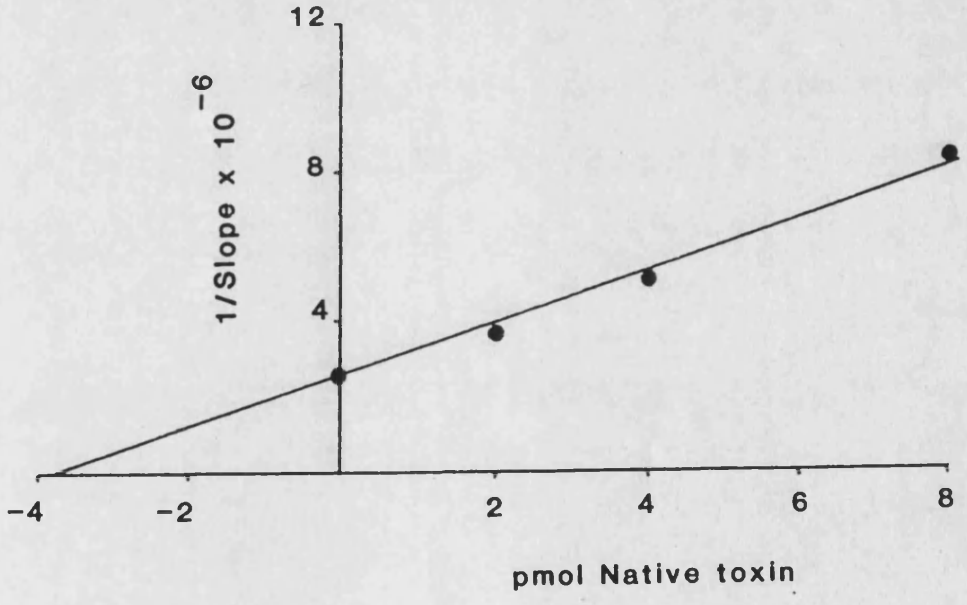

12
8
4
1/Slope x 10^{-6}
-4
-2
2
4
6
8
pmol Native toxin

Table 4.1.4 Concentration of Radiolabelled αBungarotoxin in Various Preparations: Comparison of Methods of Estimation

^{125}I α-TOXIN PREPARATION	METHOD OF CALCULATION	
	FROM GEL FILTRATION* (pmol/μl)	FROM COMPETITION STUDIES (pmol/μl)
Monoiodinated (αBGT purified from venom)	0.20	0.29
Unfractionated (αBGT purified from venom)	0.75	0.70
Unfractionated (commercial αBGT)	0.60	0.55

* Following separation of free ^{125}I from ^{125}I-αBGT on Sephadex G25 the concentration of radiolabelled toxin in fractions was calculated as described in section 3.1.3.

4.2 PURIFICATION OF Naja naja siamensis α-TOXIN (α-naja TOXIN)

The α-toxin from Naja naja siamensis venom was purified to provide an affinity ligand for the purification of acetylcholine receptor from Torpedo electric organs. The α-naja toxin was fractionated by ion-exchange chromatography on Phosphocellulose. Figure 4.2.1 shows the elution profile of Naja naja siamensis venom from the phosphocellulose column. Fractions from the column were assayed for their ability to inhibit the binding of iodinated α-bungarotoxin to Torpedo AChR. Peak 2 had the greatest inhibition activity. Recoveries from the peaks are shown below.

Peak 1	50.3mg	5% of original protein
Peak 2	103.8mg	10% of original protein

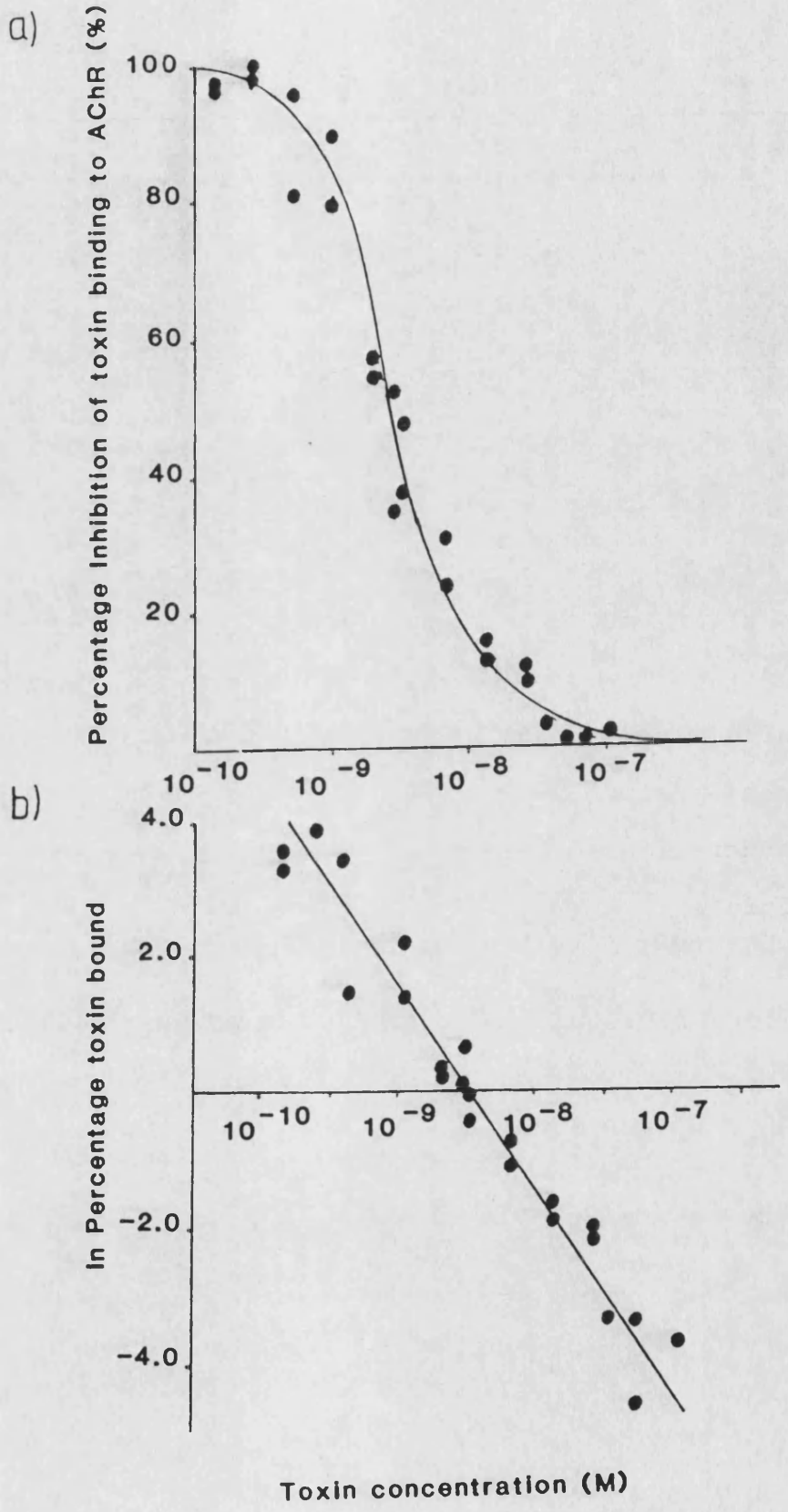
The IC_{50} values of both peaks were measured by adding increasing amounts of the toxin to fixed amounts of AChR and iodinated αBGT and measuring the inhibition of binding. Examples of the results are shown in Figures 4.2.2 a and b. The IC_{50} of peaks 1 and 2 were 5.4 x 10^{-4}M and 9.8 x 10^{-7}M respectively. The -toxin, peak 2, was lyophilised and coupled to Sepharose 4B.

Figure 4.2.2 Determination of the IC_{50} of purified α-Naja toxin

a) Percentage inhibition of αBGT binding to AChR. Increasing amounts of the α-Naja toxin were coincubated with a fixed amount of AChR and iodinated αBGT and the assay performed as described in methods 3.1.6(i). Percentage inhibition of αBGT binding to AChR was plotted against the concentration of α-Naja toxin added.

b) ln Percentage toxin bound. The natural log of the percentage of α-Naja bound was plotted against the concentration of α-Naja added. The intercept on the horizantal axis gives the IC_{50} of the toxin.

A value of 9.8×10^{-7} was obtained by linear regression.


a)
Percentage Inhibition of toxin binding to AChR (%)
100
80
60
40
20
10^{-10}
10^{-9}
10^{-8}
10^{-7}
b)
ln Percentage toxin bound
4.0
2.0
-2.0
-4.0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
Toxin concentration (M)

4.3 PURIFICATION OF TORPEDO ACETYLCHOLINE RECEPTOR

4.3.1 Purification of Torpedo Acetylcholine Receptor on an α-Toxin Affinity Column

Torpedo AChR was essentially purified by detergent extraction and affinity chromatography as described by Lindstrom (1976). The specific activity of the receptor preparations was in the range 0.6 - 6.1, mean = 3.0 pmol/μg (n = 8). Modifications of the general method of purification were carried out in order to effect an improvement in the yields and specific activity of receptor preparations. Results can be seen in Table 4.3.1. Elution of the receptor from the affinity gel using benzoquinonium chloride (10mM, see section 3.3.2) produced receptor preparations with specific activities in the range of 1.0 - 10.7, mean = 3.5 (n = 6). The specific activity of receptor preparations was not generally improved by the use of 2% Triton X100 for solubilisation. Specific activities (for receptor solubilised with 2% Triton X100) were 1.9 - 5.6 (mean = 3.3, n = 10) and 1.7 - 7.4 (mean = 3.5, n = 16) for receptor eluted from the affinity column by using carbachol and benzoquinonium chloride respectively. Elution of the receptor from the affinity gel by using carbachol always resulted in higher yields of protein and toxin binding activity. The specific activity was apparently unaffected by the overall time of

Table 4.3.1 Specific Activities of Torpedo Acetylcholine Receptor Preparations

METHOD OF PURIFICATION	PROTEIN (μg/ml)	TOXIN BINDING (pmol/ml)	SPECIFIC ACTIVITY (pmol/μg)	PROTEIN/100g ORIGINAL TISSUE (μg/100g)	BINDING/100g ORIGINAL TISSUE (pmol/100g)	TOTAL PROTEIN (mg)	n
1	1531 (880)	4146 (1220)	3.0 (0.6)	4.7 (1.6)	14751 (5345)	12.8 (3.1)	8
2	1009 (219)	3612 (700)	3.5 (0.8)	1.6 (0.9)	12547 (1694)	3.6 (0.9)	6
3	2174 (853)	6289 (1986)	3.3 (0.3)	9.8 (2.9)	34367 (338)	23.7 (6.4)	10
4	1073 (198)	3442 (735)	3.5 (0.4)	3.6 (0.6)	12061 (1997)	11.4 (1.8)	16
5	263 (60)	536 (194)	1.9 (0.3)	0.7 (0.1)	1335 (2300)	2.2 (0.4)	3

Figures in brackets represent the standard error of mean.

1. Basic method of purification, essentially as described by Lindstrom (1976).
2. Basic method using benzoquinonium for elution of the AChR from affinity column.
3. Basic method using 2% Triton X100 for solubilisation of the receptor.
4. As (3) using benzoquinonium chloride for elution.
5. Purification using anti-(Torpedo AChR) antibody affinity column, modified method of Lennon et al., 1980.

purification.

Percentage recoveries of acetylcholine receptor from the crude Triton extract were in the region of 10 - 70% for all the preparations. Percentage recoveries of receptor were greatest when carbachol was used to elute the receptor from the affinity column. Overall yields of protein were greatest when 2% Triton X100 was used for solubilisation. Similarly, elution of the AChR from the affinity column using carbachol resulted in higher yields than when benzoquinonium chloride was used. However, the use of benzoquinonium chloride increased the speed of purification.

4.3.2 Purification of Torpedo Acetylcholine Receptor on an Anti-(Torpedo AChR) Monoclonal Antibody Affinity Column

AChR was purified essentially as described in method 3.3.3 with the exception that the receptor was purified from the Triton extract by using an affinity column of anti-(Torpedo AChR) monoclonal antibodies. Receptor purified by this method gave a much lower yield and specific activity than with the conventional α-Naja toxin affinity column. The specific activity of the receptor purified on the antibody affinity column was approximately 18% of the best value obtained by the conventional method. Yields of receptor from the Triton extract were in the range of 6 - 11% and were

insufficient to allow for studies on the carbohydrate nature to be carried out. Therefore, the AChR was routinely purified by α-toxin affinity chromatography.

A summary of the results of purification of acetylcholine receptor by affinity chromatography on the monoclonal antibody affinity column is shown in Table 4.3.1.

4.3.3 Effect of Gel Filtration on the Torpedo AChR

Further purification of Torpedo acetylcholine receptor, after ion-exchange chromatography, was attempted by subjecting purified AChR (concentrated on a Minicon concentrator) to gel filtration on a column of Ultragel ACA 34 or Sephacryl S200. Fractions from the columns were assayed for toxin binding activity and protein content (Lowry). The protein peak corresponding to the AChR was pooled and assayed as before. The mean specific activity of seven AChR preparations was 3.0 (S.E.M = 0.08). After gel filtration, this value was increased to 5.0 (S.E.M = 0.37). In some instances the specific activity of AChR preparations was increased by approximately 100% following gel filtration.

An example of the elution profile of the receptor on Sephacryl S200 is shown in Figure 4.3.1 in which a small but discrete protein peak, separate from the AChR peak, is evident. This corresponds to low molecular weight proteins possibly resulting from proteolysis of

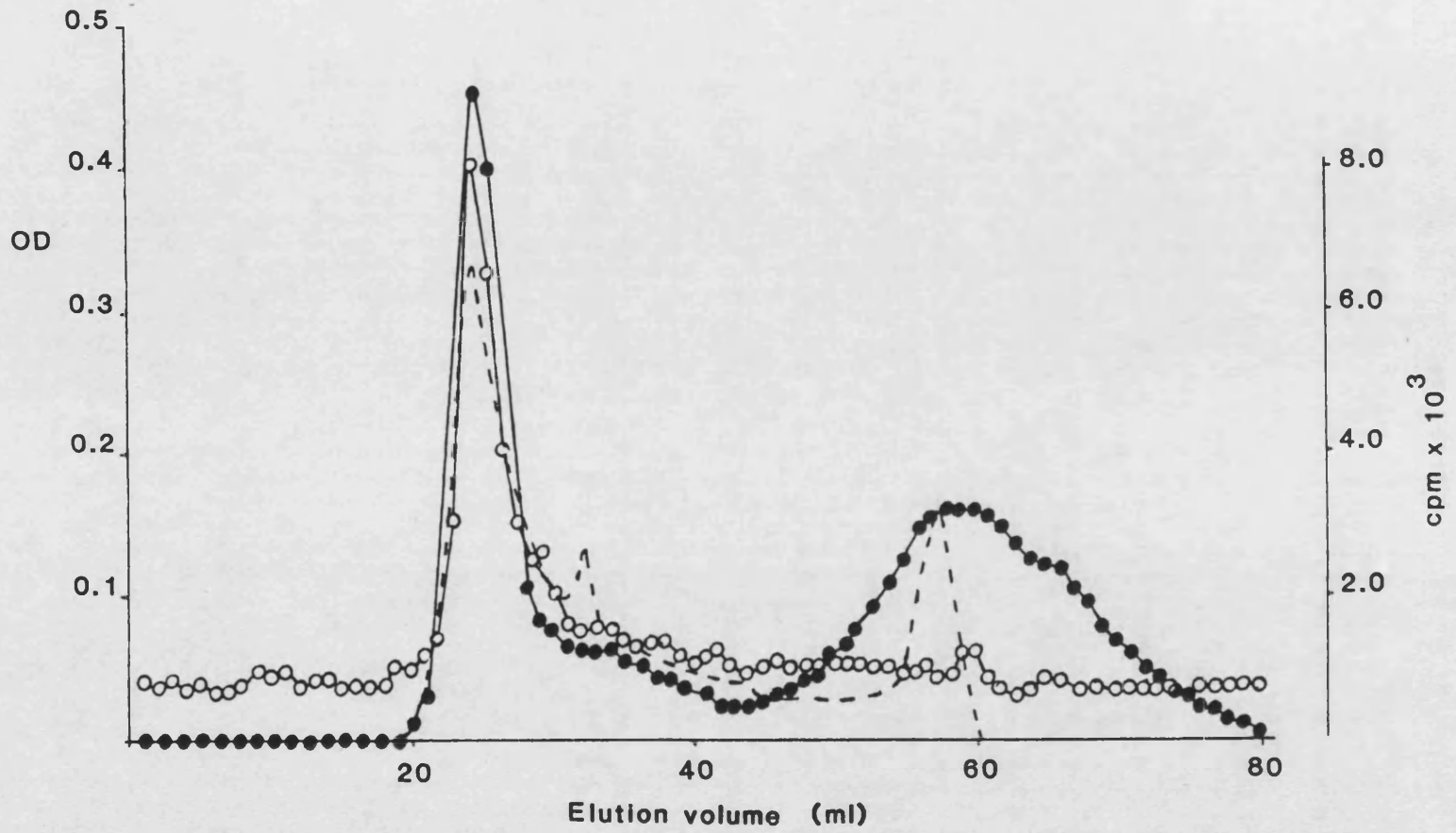


Figure 4.3.1 Chromatography of AChR (1.5 - 2.5 mg) on Sephacryl S200 (superfine) column (1.5cm x 30cm). The column was eluted with potassium phosphate buffer, 10mM, pH 7.4 containing 0.3M NaCl. Fractions of 1ml were collected with a flow-rate of 60ml/h. (●) Protein (280nm), (- - -) protein (Lowry et al, 1951), (o) toxin binding activity.

AChR. This extra protein peak constituted (in one instance) approximately 18% of the total protein applied to the column.

The elution profile of the acetylcholine receptor, as measured at 280nm, shows a broad peak around the area for the small molecular weight proteins. This broad peak is a result of nonbound Triton X100 which also absorbs light at this wavelength.

4.4 CHEMICAL ANALYSIS OF AChR CARBOHYDRATE MOIETY

All AChR preparations were subjected to gel filtration on Ultragel ACA 34 or Sephacryl S200 before any carbohydrate analysis was carried out. Following chromatography the purity of preparation were checked by SDS PAGE.

4.4.1 Sialic Acid Content of Intact Torpedo AChR

It has been reported that Torpedo AChR contains terminal sialic acid residues. In order to confirm this sialic acid was assayed in the intact receptor.

Results from the thiobarbituric acid assays are presented in Table 4.4.1. The mean mole/mole ratio of sialic acid to protein (assuming a molecular weight of the receptor to be 250,000) for untreated AChR is 1.03 (0.13% sialic acid).

Table 4.4.1 Sialic Acid Content of the AChR and the Effect of Neuraminidase Treatment

AChR PREPARATION	MOLAR RATIO (NeuNAc/AChR)		PERCENTAGE NeuNAc in AChR		NeuNAc/mg PROTEIN(μg/mg)		REDUCTION in NeuNAc (%)
	before	after	before	after	before	after	
1	0.90	N.D	0.11	N.D	1.14	N.D	100
2	0.58	0.27	0.07	0.03	0.71	0.33	53.5
3	1.71	-	0.21	-	2.12	-	-
4	0.91	-	0.14	-	1.36	-	-
5	0.84	0.46	0.10	0.04	1.05	0.69	45.3

N.D. Not detected.
- Assay not performed.
before/after - before/after neuraminidase treatment

4.4.2 Effect of Neuraminidase Treatment on the Sialic Acid Content of the Acetylcholine Receptor

Sialic acid was removed from AChR by incubation with neuraminidase. The sialic acid was separated from intact Torpedo receptor by gel filtration on Sephadex G25. Fractions were assayed for protein, sialic acid and hexose, see Figure 4.4.1. The elution profile of the neuraminidase treated acetylcholine receptor shows that only the sialic acid was cleaved from the AChR. Hexose sugars were found to be eluted with the receptor protein. 50 - 100% of the sialic acid was removed by neuraminidase treatment. Results of the sialic acid assays are shown in Table 4.4.1.

4.4.3 Hexose and Hexosamine Content of Torpedo AChR

Sugar residues were cleaved from intact Torpedo AChR by acid hydrolysis. These were then chemically modified to produce their alditol acetate derivatives which were then subjected to gas-liquid chromatography. Standard mixtures of sugars were similarly treated and used to identify sugars from Torpedo acetylcholine receptor. The chromatographic trace obtained from injections of the standard sugars onto the column of OV 225 is shown in Figure 4.4.2.

Some samples of purified AChR, when derivatised, produced a varying number of peaks on the OV 225 column. Only some of these peaks could be identified from the

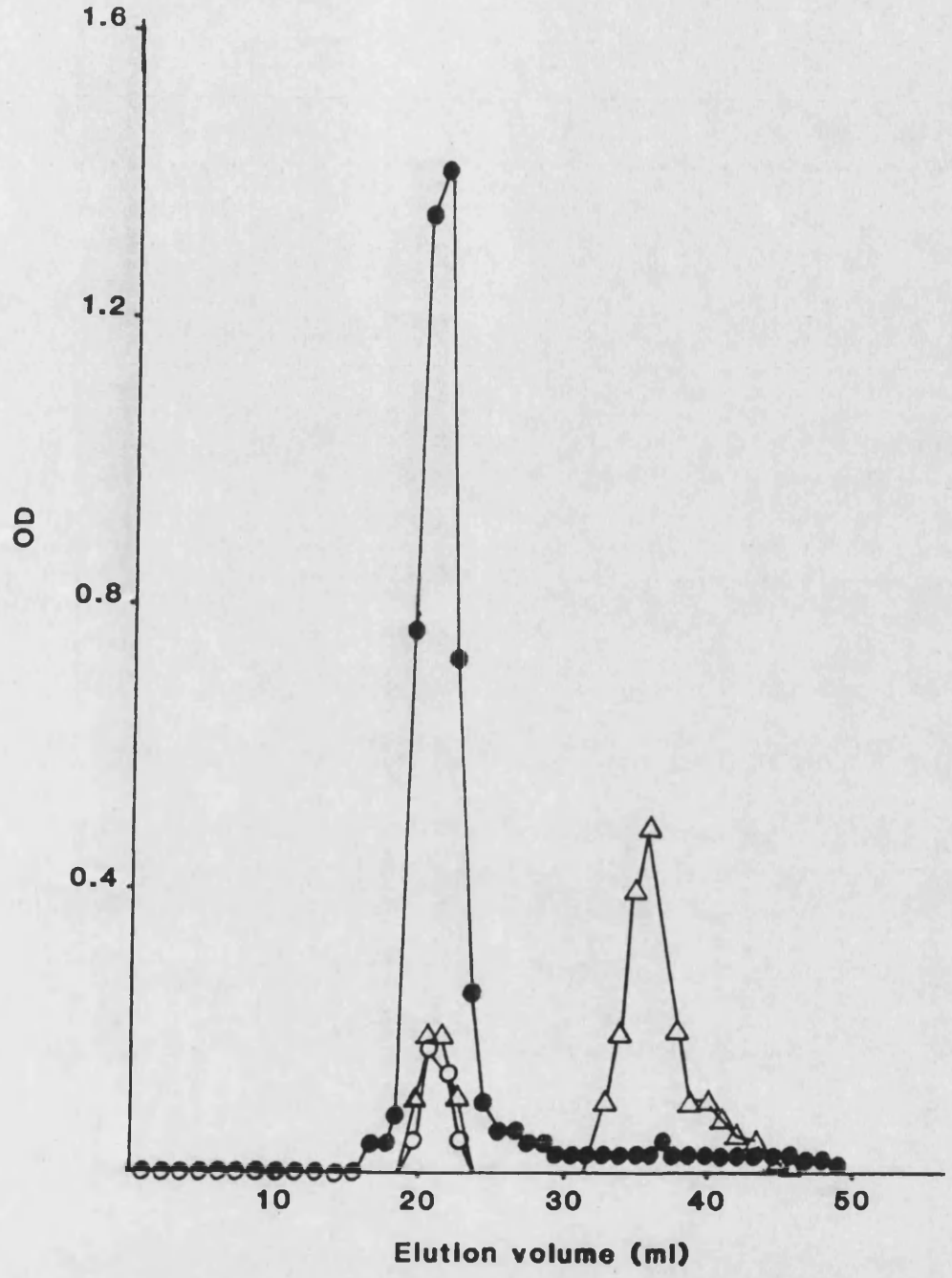


Figure 4.4.1 Elution profile of neuraminadase treated AChR on Sephadex G25 column (1.8cm x 82cm). The column was eluted with 10mM ammonium acetate, pH 7.4, and fractions of 3ml were collected with a flow-rate of 30ml/h. (●) Protein (Lowry et al., 1951), (o) hexose sugars (cysteine/sulphuric acid assay), (Δ) sialic acid (thiobarbituric acid assay).

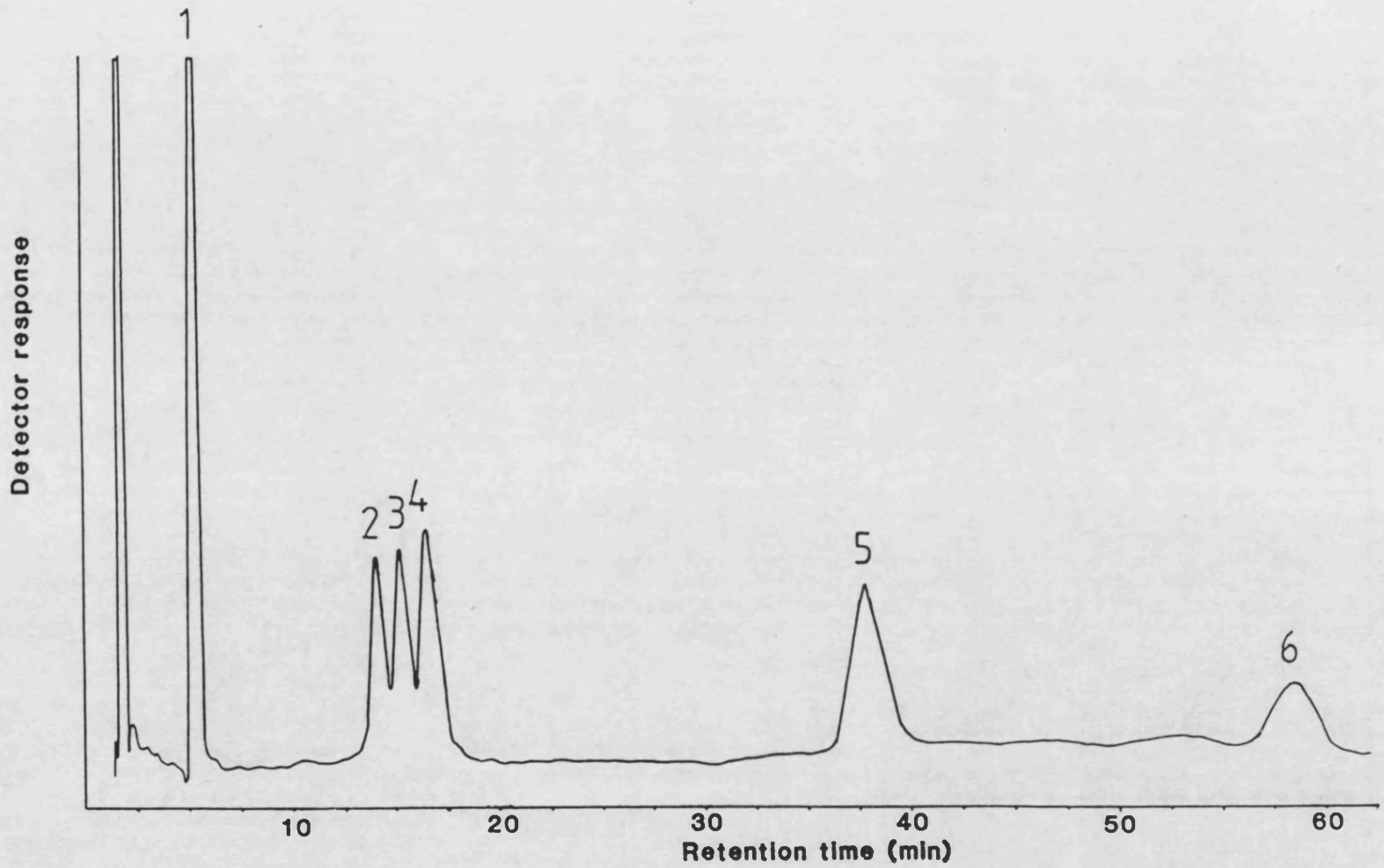


FIGURE 4.4.2 Gas chromatography of alditol acetate derivatised monosaccharides of a standard mixture of sugars. Peaks 1 - 6 correspond to L-fucose, D-mannose, D-galactose, D-glucose, perseitol (internal standard), N-acetyl-D-glucosamine. Chromatography was performed isothermally at 21^{o}C on a column of 3% OV225.

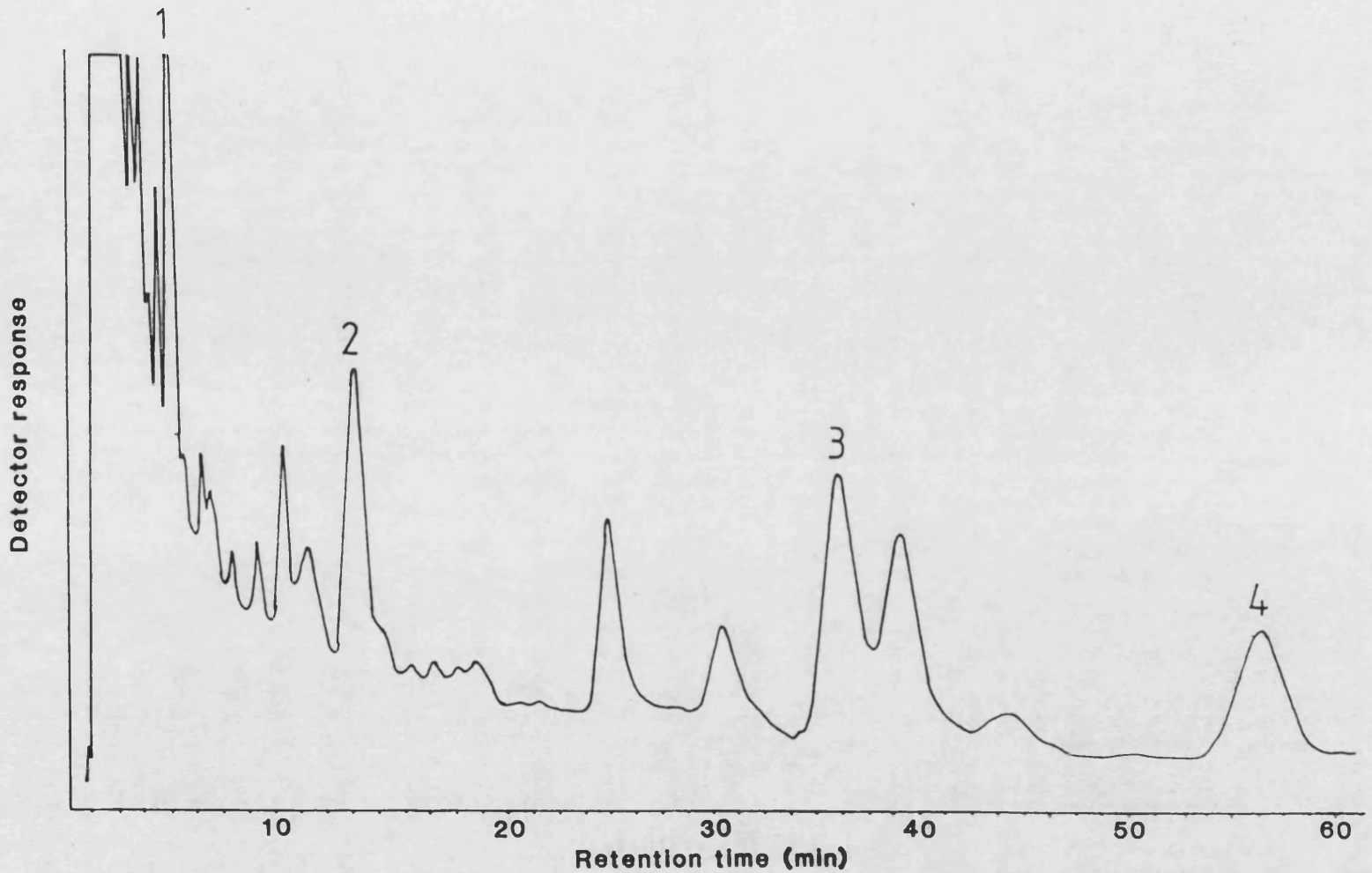
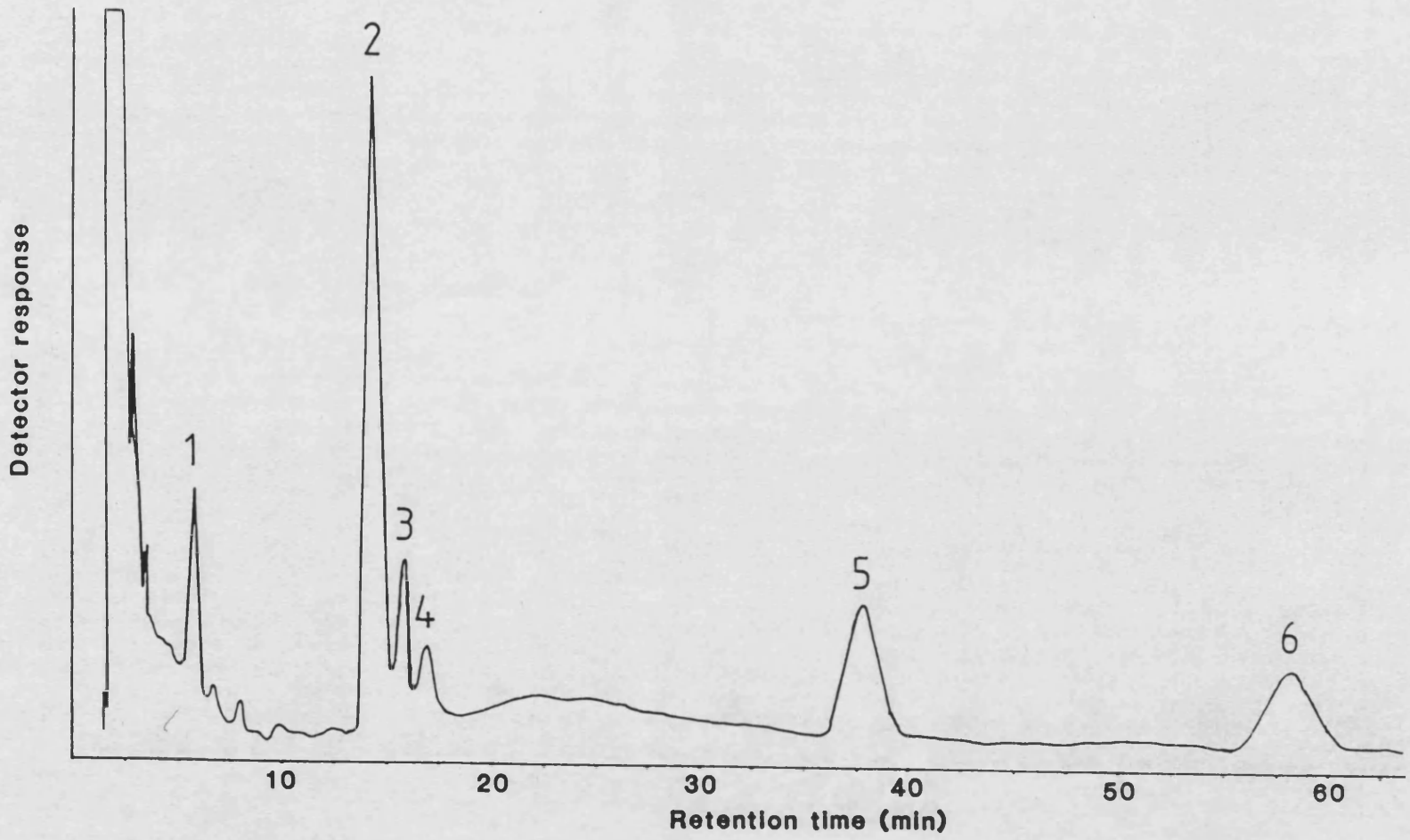


FIGURE 4.4.3 Gas chromatography of alditol acetate derivatised monosaccharides released during acid hydrolysis of purified AChR. Peaks 1 - 4 correspond to L-fucose, D-mannose, perseitol (internal standard) & N-acetyl-D-glucosamine. Chromatography was performed isothermally at 210°C on a column of 3% OV225.

FIGURE 4.4.4 Gas chromatography of alditol acetate derivatised monosaccharides released during acid hydrolysis of Torpedo AChR after chromatography on Sephacryl S200. Peaks 1 - 6 correspond to L-fucose, D-mannose, D-galactose, D-glucose, perseitol (internal standard) and N-acetyl-D-glucosamine. Chromatography was performed isothermally at 210^{o}C on a column of 3% OV225.


Detector response
1
2
3
4
5
6
10
20
30
40
50
60
Retention time (min)

trace (Figure 4.4.3). When the AChR was subjected to column chromatography on ACA 34 or Sephacryl S200 and then derivatised as before virtually all the extraneous peaks were removed (Figure 4.4.4). Consequently AChR preparations were always subjected to column chromatography prior to derivatisation for gas-liquid chromatography. The purity of the acetylcholine receptor preparations was also checked by SDS polyacrylamide gel electrophoresis (see section 4.5.4).

Sugars that were always present on gas chromatography of AChR were L-fucose, D-mannose, D-galactose and N-acetyl-D-glucosamine. D-glucose was not always evident and, when present, this peak was very variable in height.

The sugar contents of acetylcholine receptor preparations are shown in Tables 4.4.2 - 4.4.5. These tables also show the results of enzyme and alkaline borohydride treatment. The glucose peak was highly variable and may in fact be a contaminant. The molar ratios of the individual monosaccharides from Torpedo acetylcholine receptor were 1.4/3.2/1/0.3/1.1 for fucose, mannose, galactose, glucose and N-acetylglucosamine. 6.3 $\pm$ 0.6% (by weight, n = 10) of the acetylcholine receptor was found to consist of sugar with a total of 87 $\pm$ 12 (n = 10) sugar residues per receptor molecule. The number of sugar residues per receptor molecule was identical regardless of whether it was determined from the specific toxin binding or the protein content (Lowry).

No trace of sugar residues was found in samples of

Table 4.4.2 Monosaccharide Content of Treated and Untreated Acetylcholine Receptor from Torpedo marmorata

RECEPTOR TREATMENT	MONOSACCHARIDE COMPONENT (ng/μg)					AMOUNT OF SUGAR IN TORPEDO AChR (%)	n
	L-FUCOSE	D-MANNOSE	D-GALACTOSE	D-GLUCOSE	N-ACETYL-D-GLUCOSAMINE		
UNTREATED	11.6 (2.5)	26.8 (2.7)	10.3 (1.7)	5.1 (2.4)	9.7 (3.5)	6.3 (0.6)	10
ALKALINE BOROHYDRIDE	749	2029	701	-	640	380	1
ENDO-GLYCOSIDASE	7.2 (0.2)	13.2 (1.9)	3.9 (0.8)	5.5 (4.9)	5.9 (0.8)	3.5 (0.5)	3
T.FOETUS	7.4 (0.3)	21.2 (3.9)	5.9 (0.7)	-	5.7 (2.0)	2.8 (1.3)	3

Figures in brackets represent the standard error of mean.

Table 4.4.3 Molar Ratios of Monosaccharides from Torpedo Acetylcholine Receptor

RECEPTOR TREATMENT	MONOSACCHARIDE COMPONENT				
	L-FUCOSE	D-MANNOSE	D-GALACTOSE	D-GLUCOSE	N-ACETYL-D-GLUCOSAMINE
UNTREATED	1.4 (0.2)	3.2 (0.4)	1	0.3 (0.1)	1.1 (0.3)
ALKALINE BOROHYDRIDE	1.2	2.9	1	-	0.4
ENDO-GLYCOSIDASE	3.5 (0.4)	3.1 (0.1)	1	1.3 (0.6)	0.7 (0.1)
T.FOETUS	1.0 (0.1)	3.2 (0.3)	1	-	0.9 (0.2)

Figures in brackets represent standard error of mean.

Number of samples:- Untreated = 10, alkaline borohydride treated = 1, enzyme treated = 3 each.

Table 4.4.4 Monosaccharide Content of Treated and Untreated Acetylcholine Receptor from Torpedo marmorata (nmol/mg)

RECEPTOR TREATMENT	MONOSACCHARIDE COMPONENT (nmol/mg)					
	L-FUCOSE	D-MANNOSE	D-GALACTOSE	D-GLUCOSE	N-ACETYL-D-GLUCOSAMINE	TOTAL
UNTREATED	47.1 (4.0)	122.7 (16.4)	40.3 (7.0)	21.3 (9.4)	41.8 (7.4)	263 (23.1)
ALKALINE BOROHYDRIDE	4560	11260	3888	-	1444	21152
ENDO-GLYCOSIDASE	41.6 (0.8)	76.3 (3.1)	22.5 (1.2)	30.6 (15.3)	27.5 (1.2)	187 (28.2)
T.FOETUS	40.6 (1.6)	104.4 (18.2)	24.4 (6.0)	-	29.2 (4.8)	209 (25.3)

Figures in brackets represent the standard error of mean.

Number of samples:- Untreated = 10, alkaline borohydride treated = 1, enzyme treated = 3 each.

Table 4.4.5 Carbohydrate Content of Torpedo Acetylcholine Receptor (Molar Ratio Sugar/AChR)

MONOSACCHARIDE COMPONENT					
L-FUCOSE	D-MANNOSE	D-GALACTOSE	D-GLUCOSE	N-ACETYL-D-GLUCOSAMINE	TOTAL
13.1	35.4	9.9	9.0	9.9	86.0
S.E.M ± 2.1	± 7.4	± 2.3	± 4.2	± 2.1	± 16.0
11.9	33.8	12.2	7.1	11.6	88.4
S.E.M ± 1.5	± 4.8	± 2.0	± 3.3	± 2.3	± 7.7

The first set of figures is calculated from the specific toxin binding of the acetylcholine receptor preparations and the second set from the protein concentration. n = 10

buffer eluted through any of the columns used in the purification of the acetylcholine receptor from Torpedo.

4.4.4 Enzyme Treatment of Torpedo AChR

To verify that carbohydrates found by gas chromatography were indeed from purified Torpedo AChR, the receptor was treated with various enzymes (mixed endoglycosidase D and T.foetus enzymes) to remove bound sugars (see section 3.5.4 and 3.5.5.

Mixed endoglycosidase D or T.foetus enzymes were separated from the AChR by gel filtration on Sephacryl S200. Fractions from the column were assayed for protein (Lowry, 1951), glycosidase activity and toxin binding activity The elution profile of T.foetus treated AChR is shown in Figure 4.4.5. The acetylcholine receptor peak was pooled derivatised and subjected to gas-liquid chromatography as in Section 4.4.2.

4.4.5 GLC Analysis of the Carbohydrate Content of Enzyme Treated AChR

Enzyme treated acetylcholine receptor was subjected to gas-liquid chromatography as before (see Tables 4.4.2 - 4.4.5). Changes in the monosaccharide content of the AChR with enzyme treatment are summarised in Table 4.4.6.

Gas chromatography of the mixed endoglycosidase D treated AChR showed that there was a significant reduction ($P = 0.5$) of 44% in the total weight of sugar

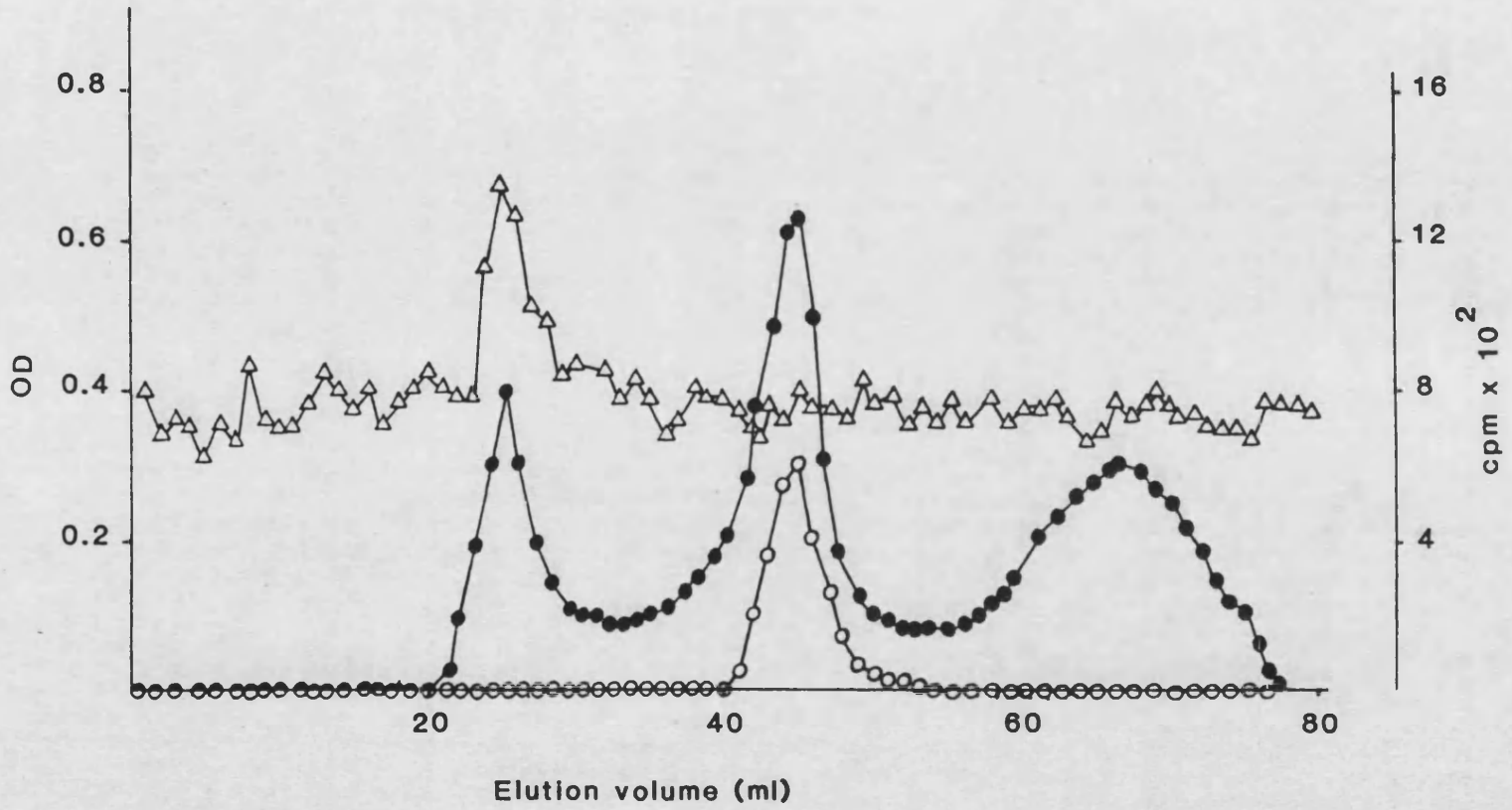


Figure 4.4.5 Chromatography of T. foetus treated AChR on Ultragel ACA 34 column (1.2cm x 51cm) using potassium phosphate buffer (10mM), pH 7.4, containing 0.3M NaCl. Eluates of 1ml were collected with a flow-rate of 12ml/h. (O) Glycosidase activity (Distler & Jourdian, 1973) (●) protein (280nm), (△) toxin binding activity.

Table 4.4.6 Percentage Reduction in Monosaccharide Content of Acetylcholine Receptor Treated with Glycosidases

ENZYME TREATMENT	MONOSACCHARIDE COMPONENT					
	L-FUCOSE	D-MANNOSE	D-GALACTOSE	D-GLUCOSE	N-ACETYL-D-GLUCOSAMINE	TOTAL
ENDO-GLYCOSIDASE	37.9	50.7	62.1	60.0*	39.5	44.0[1]
T.FOETUS	36.2	20.9	42.7	100	41.2	55.5[2]

Number of determinations for each enzyme treatment = 3

* Percentage increase

Significant at [1] P = 0.5, [2] P = 0.01.

as compared with the untreated receptor. There was a reduction of 37.9%, 50.7%, 62.1% and 39.5% of fucose, mannose, galactose and N-acetylglucosamine respectively. There was an apparent 60% increase in the glucose content.

T.foetus treatment, as with the previous enzyme treatment, resulted in a significant decrease ($P = 0.01$) in the percentage of the total sugars (by weight). There was a 55.5% reduction in the total sugars, 36.2%, 20.9%, 42.7%, 100% and 41.2% from fucose, mannose, galactose, glucose and N-acetyl-D-glucosamine respectively.

Endoglycosidase treatment only significantly altered the molar ratios of fucose to galactose ($P = 0.1$) and glucose to galactose ($P = 2.5$) as compared with the untreated acetylcholine receptor. T.foetus treatment had no such significant effect.

4.4.6 Alkaline Borohydride Treatment of Torpedo Acetylcholine Receptor

Alkaline borohydride treated AChR was run on a Sephadex G25 column and fractions were assayed for protein, hexose and toxin binding activity. All toxin binding was eliminated by the alkaline borohydride treatment. This may have been a result of the complete breakdown of the protein as evident from the elution profile. All the hexoses appeared to have remained bound to the protein as none was found to be included in the

column. Figure 4.4.6 shows the elution profile.

4.4.7 GLC Analysis of the Carbohydrate Content of Alkaline Borohydride Treated AChR

Alkaline borohydride treated AChR resulted in an apparent increase in the percentage of sugar in the receptor (see Tables 4.4.3 - 4.4.6). This, however, results from the breakdown of the receptor complex. A glucose peak was absent from gas chromatography traces of the treated receptor. The ratios of sugars were approximately the same as in the untreated acetylcholine receptor.

4.5 GEL ELECTROPHORESIS AND ISOELECTRIC FOCUSSING

4.5.1 Effect of the Removal of Carbohydrate Residues on the P_i of AChR

Acetylcholine receptor was incubated with mixed endoglycosidases D for 60, 90, 120 and 240 min. Neuraminidase treatment was also carried out on the receptor. The enzymes were separated from the receptor protein by gel filtration on Sephacryl S200. Treated and untreated AChR were incubated with iodinated αBGT and subjected to isoelectric focussing. Typical profiles on isoelectric focussing gels are shown in Figures 4.5.1 and 4.5.2.

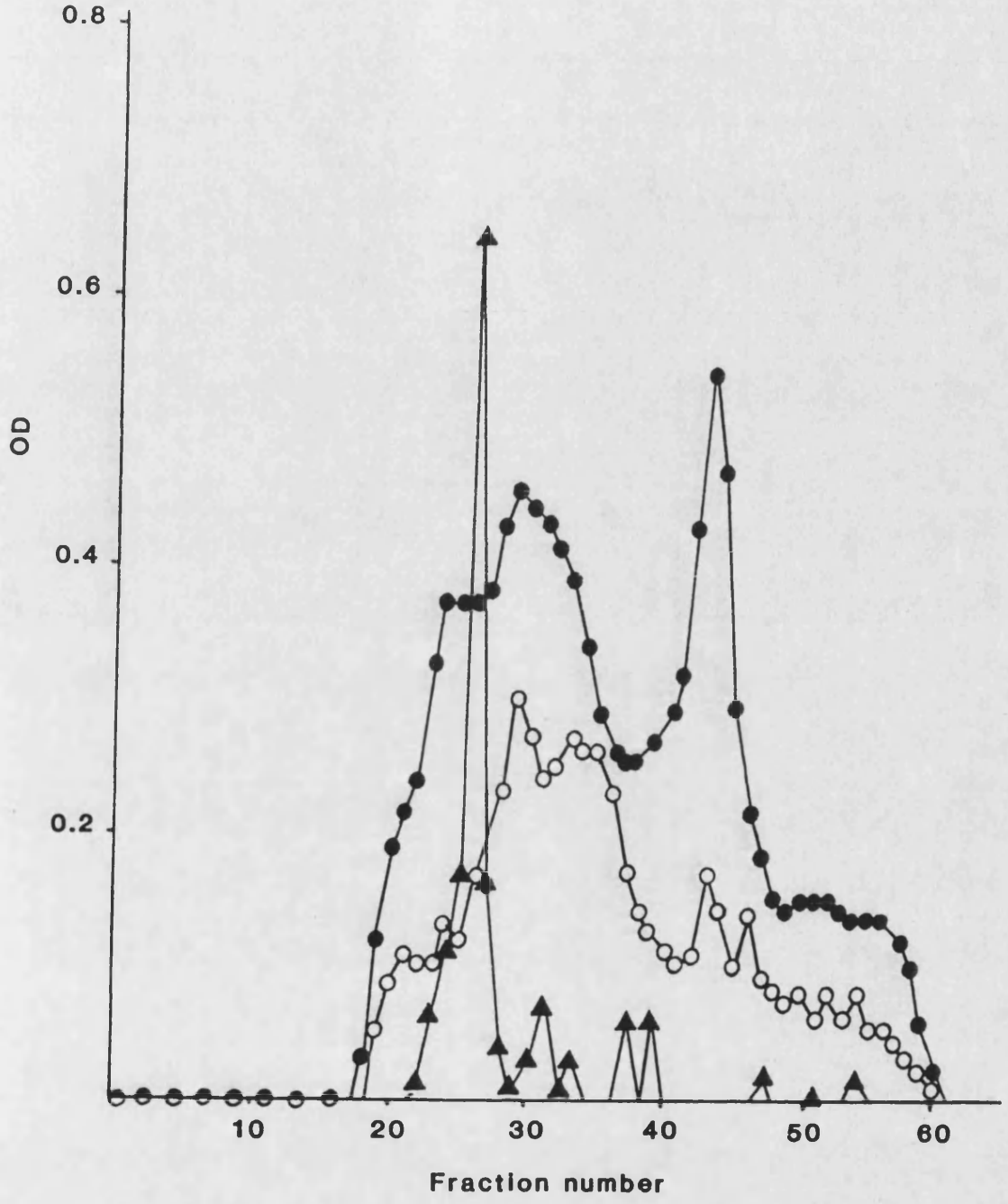


Figure 4.4.6 Elution profile of alkaline borohydride treated AChR on Sephadex G25 column (1.8cm x 82cm) using 10mM ammonium acetate buffer, pH 7.4. Eluates of 3ml were collected with a flow-rate of 30ml/h. (●) Protein (280nm), (o) protein (Lowry et al., 1951), (▲) hexose sugars.

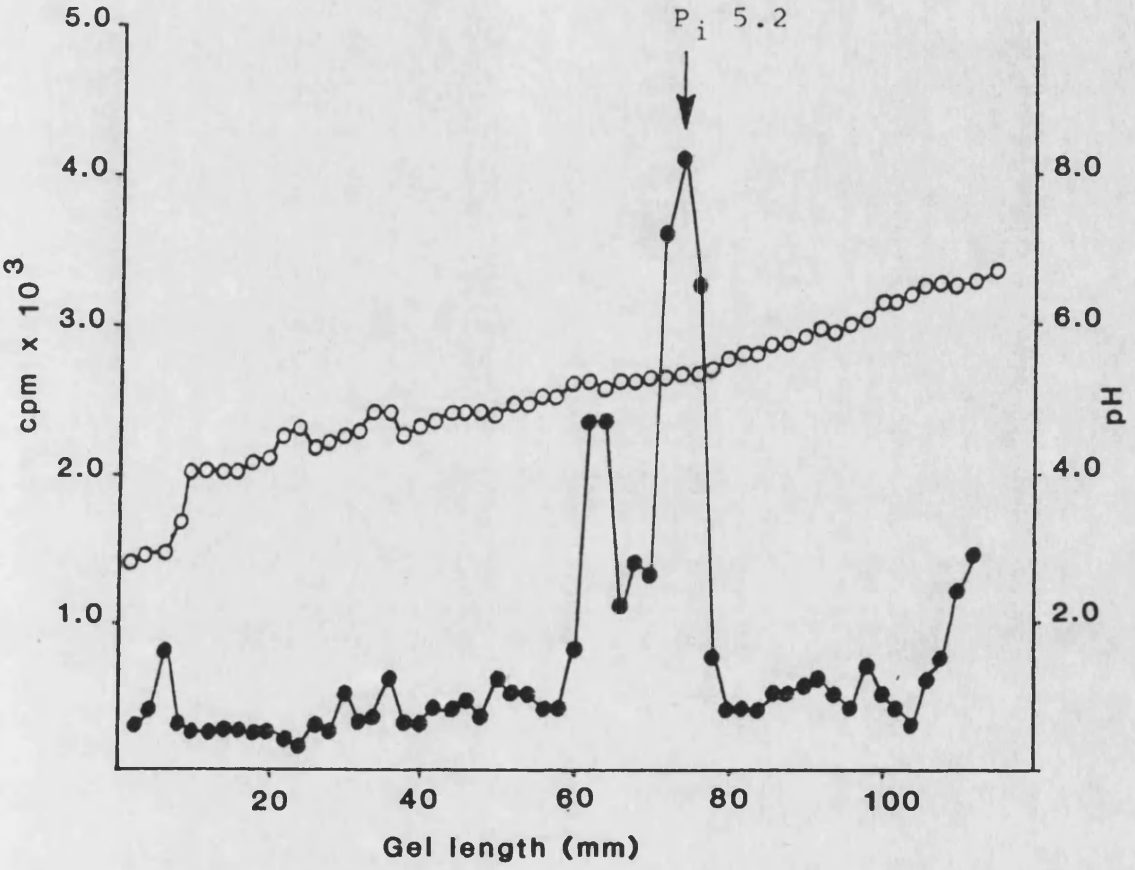


Figure 4.5.1 Isoelectric focusing gel of AChR following incubation with ^{125}I-αBGT.

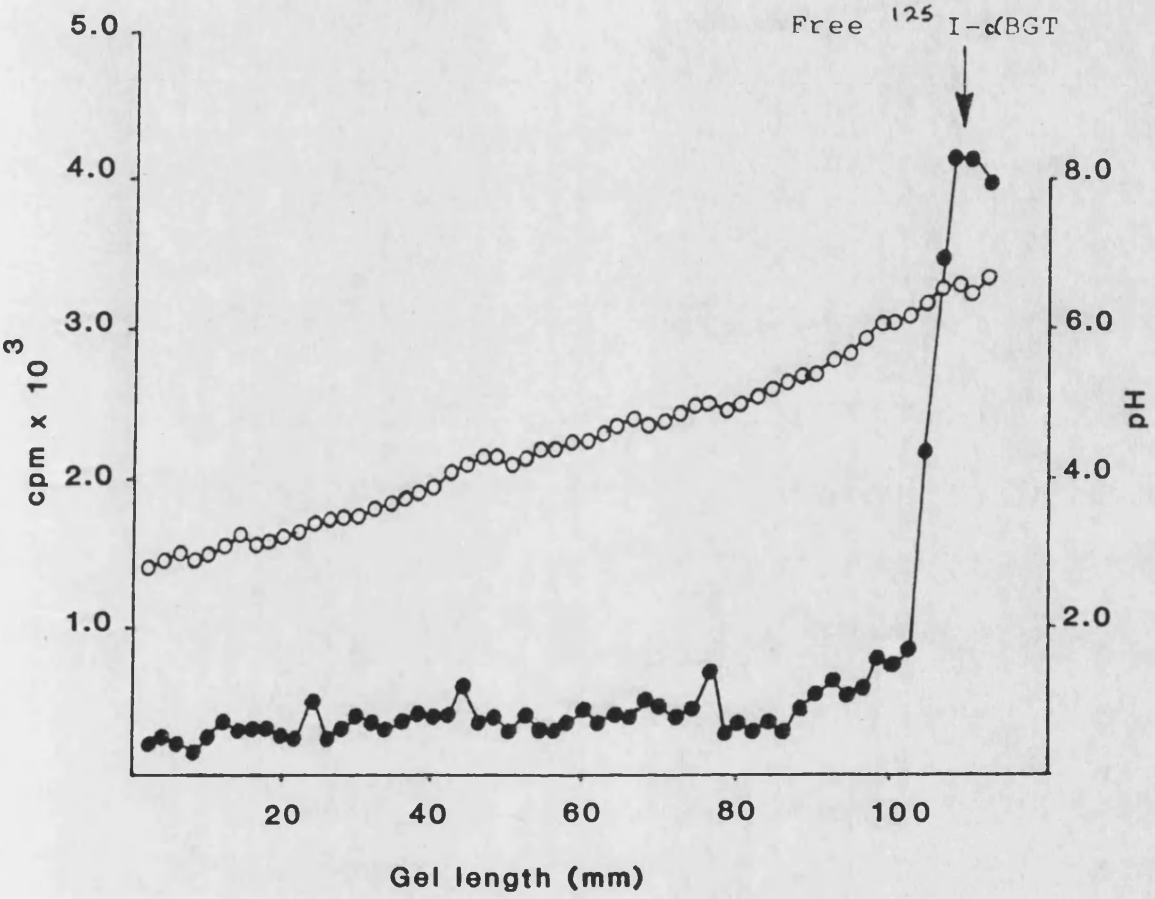


Figure 4.5.2 Isoelectric focusing gel of AChR following incubation with ^{125}I-αBGT in the presence of 25mM benzoquinonium chloride.

Untreated AChR labelled with iodinated αBGT gave an isoelectric point of 5.3 (S.E.M = ± 0.12, n = 4). Treated AChR gave the values shown in Table 4.5.1.

There is no significant difference (t test) between the P_i values of the untreated and treated AChR. However, these results should be treated with caution as the extremely electropositive nature of the ^{125}I-αBGT may mask any apparent change in the P_i value.

4.5.2 Protein Staining of SDS Polyacrylamide Gels: Comparison of Methods

Two methods of staining gels for protein were investigated. Standard proteins were run on a gradient (5 - 14% w/v) acrylamide gel. After electrophoresis the gel was cut in half and each half stained with either 2.5mg/ml Coomassie brilliant blue R 250 in 45% methanol/10% acetic acid or in 0.04% (w/v) Coomassie brilliant blue R 250 in 12.5% (w/v) TCA (both left overnight). Destaining was carried out in 30% methanol/10% acetic acid and 12.5% TCA respectively.

The methods of staining seemed to be equally sensitive. However, when the gel is stained in TCA, the bands were visible before destaining commences. The bands also appeared stronger. The TCA method was routinely used for staining gels.

Table 4.5.1 Effect of Enzyme Treatment on the Isoelectric Focussing Point of Torpedo Acetylcholine Receptor - αBGT Complex

ENZYME TREATMENT	LENGTH OF TREATMENT (min)	ISOELECTRIC POINT
Mixed Endoglycosidase D	60	5.0
	90	4.7
	120	5.4
	240	5.1
Neuraminidase	120	5.4

4.5.3 Carbohydrate Staining of Gels

SDS polyacrylamide slab gels (Laemmli, 1970) were stained with PAS stain to detect glycoproteins. All four subunits (from protein staining) were shown to be glycosylated (Figure 4.5.3). Molecular weights of the subunits of the PAS stained gels were within the range determined below.

4.5.4 Gel Electrophoresis: Purity Check and Molecular Weight Determination of AChR Subunits

Denatured AChR typically showed a gel pattern (Figure 4.5.4) of 4 subunits. High molecular weight bands (greater than 65,000) were not detected. Bands with molecular weights lower than 40,000 were occasionally detected and may be due to proteolytic activity. The molecular weights of the subunits of *Torpedo* AChR were determined from gel electrophoresis on 7.5% acrylamide gels (Laemmli, 1970). The subunit molecular weights obtained (from 5 gels) by this method were as follows:

$$\alpha = 42,300 \pm 800$$

$$\beta = 48,900 \pm 200$$

$$\delta = 57,300 \pm 500$$

$$\gamma = 61,800 \pm 500$$

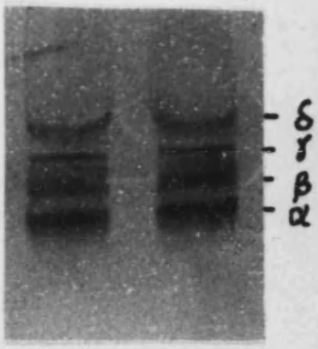


FIGURE 4.5.3 Carbohydrate staining of the subunits of Torpedo AChR. The receptor was run on SDS polyacrylamide slab gels and subsequently stained with PAS to detect glycoproteins.

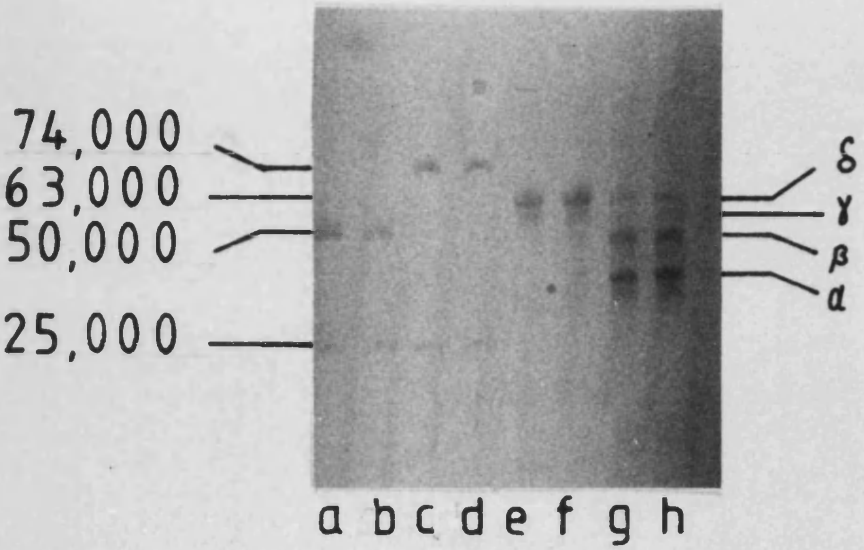


FIGURE 4.5.4 SDS polyacrylamide gel electrophoresis of Torpedo AChR. The receptor (lanes g & h) was run on 7.5% acrylamide together with standard proteins; IgG (lanes a & b), IgM (lanes c & d), BSA (lanes e & f).

4.5.5 Determination of Subunit Distribution of Torpedo AChR Carbohydrate Residues in Polyacrylamide Gels using FITC - Labelled Lectins

In order to locate specific monosaccharide residues within subunits of Torpedo AChR the receptor was run on SDS gels (Laemmli, 1970) and then incubated with FITC labelled lectins.

Acetylcholine receptor (plus a standard mixture of proteins used for the determination of subunit molecular weights) were run on 7.5% polyacrylamide gels. The gels were stained with either FITC-labelled Arachis hypogeae lectin or FITC-labelled succinylated Triticum vulgaris lectin.

When the gel stained for galactose (Arachis lectin) was viewed under long - range ultraviolet light 2 bands of the AChR (approx M.W 50,000 and 40,000) were visible. Two bands of the AChR (approx 65,000 and 60,000) were just visible on the gel stained for N-acetyl-D-glucosamine. All the bands were very faint and very difficult to photograph successfully.

This method of examining the distribution of carbohydrates in the subunits of the acetylcholine receptor proved to be very unsatisfactory and the method was not pursued any further. Therefore an alternative approach was sought (see following section).

4.6 DETECTION OF THE CARBOHYDRATE MOEITIES OF ACETYLCHOLINE RECEPTOR FROM LECTIN BINDING STUDIES USING THE ELECTROBLOT TECHNIQUE

4.6.1 Preparation of Horseradish Peroxidase / Glycoprotein Conjugate Used for the Detection of Lectin Binding to Glycoproteins Immobilised on Nitrocellulose Paper

In order to facilitate the detection of sugar residues in glycoproteins immobilised on nitrocellulose paper various glycoproteins were labelled with the enzyme, horseradish peroxidase. Three separate glycoproteins (Fc^{γ}, ovamucoid, thyroglobulin) were conjugated to horseradish peroxidase as described in section 3.9.3. The approximate concentrations of these proteins (unfractionated) are shown in Table 4.6.1.

HPO-Fc^{γ} was fractionated on Sephadex G75. Virtually all the Fc appeared to be conjugated to the HPO as shown by the elution profile (Figure 4.6.1). HPO-thyroglobulin fractionated on ACA 34 also showed very little free HPO.

4.6.2 Spot Test for Testing the Ability of Horseradish Peroxidase Labelled Glycoprotein Conjugates to Bind to Various Lectins

The HPO-conjugates were tested for their ability to

Table 4.6.1 Concentrations of Glycoprotein-HPO Conjugates

GLYCOPROTEIN	Fcγ	OVAMUCOID	THYROGLOBULIN
Weight of HPO Conjugated (mg)	8	5	5
Weight of Glycoprotein conjugated (mg)	10	3	40
Concentration of conjugated HPO (mg/ml)	1.41	1.40	1.00
Concentration of conjugated glycoprotein (mg/ml)	1.50	0.80	15.00
Molar ratio (HPO / glycoprotein)	0.94	1.75	0.07

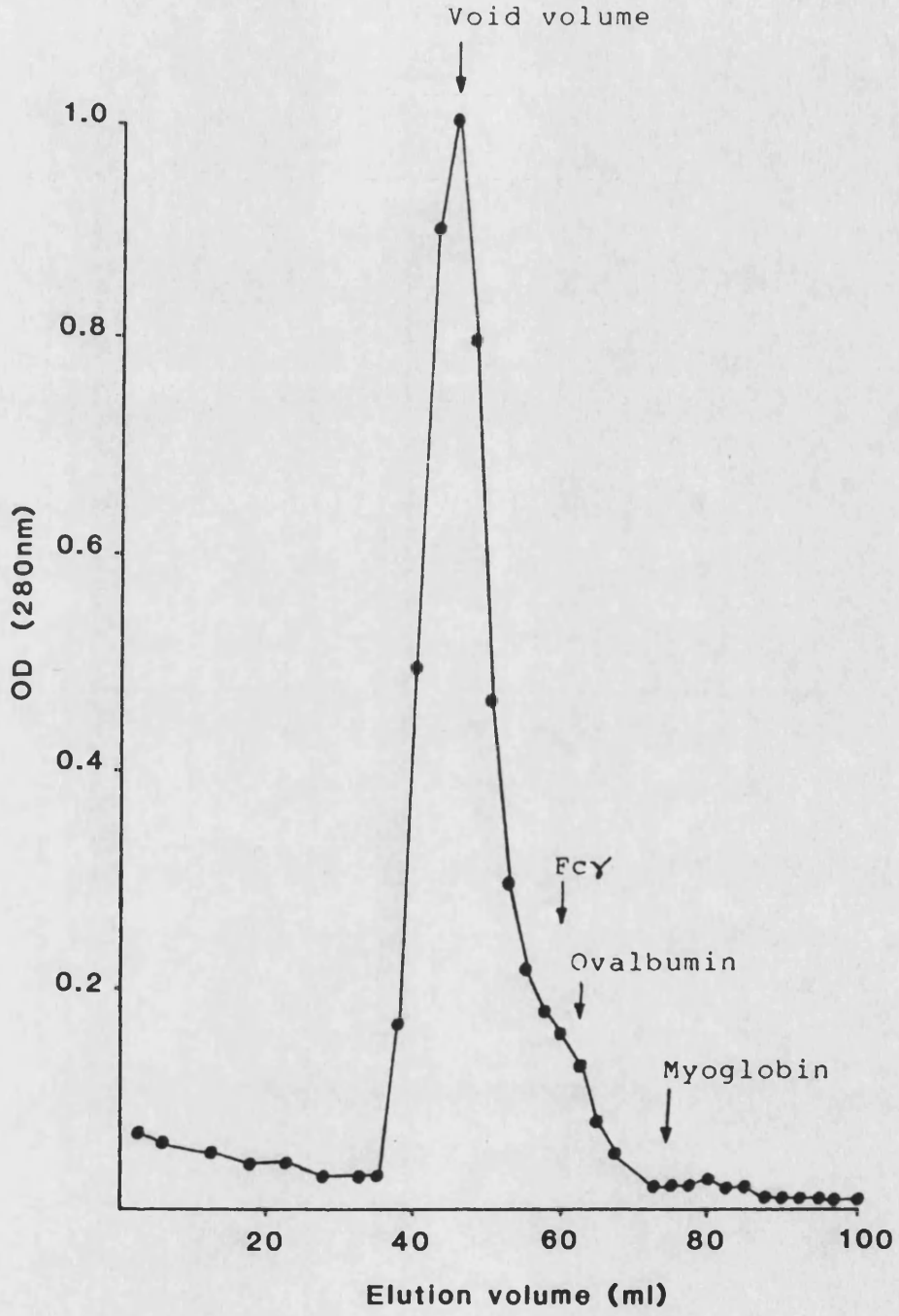


Figure 4.6.1 Elution profile of conjugated FcY-HPO on Sephadex G75 (1.5cm x 100cm). The column was eluted with PBS and fractions of 2.5ml collected.

bind lectins. Various lectins (2µg) were spotted onto nitrocellulose and allowed to dry prior to incubation with the conjugates (see section 3.9.4, i). The conjugates were diluted 1:200 and 1:400 in PBS. Incubation of the lectins with horsradish peroxidase only and with the substrate (3-amino-9-ethyl carbazole) only were used as controls. Lectins tested are shown in Table 4.6.2.

Only Con A and Pea lectins should bind to HPO (peroxidase contains only mannose) and this is confirmed by the results showing that the conjugates are specific. Only the thyroglobulin-HPO and Fcγ-HPO conjugates bound to the Ulex lectin. The binding of the Ovamucoid-HPO conjugate was generally rather poor. The greatest binding was obtained by using the Fcγ-HPO conjugate. The Fcδ-HPO conjugate produced a reaction within 3 min of incubation with the substrate. Fractionation of Fcγ-HPO made no difference to the activity of the conjugate. Dilution of the thyroglobulin-HPO and Fcδ-HPO conjugates by 1:400 still produced a very strong reaction and this conjugate dilution was used in further tests. Figure 4.6.2 shows the results of the spot test.

4.6.3 Lectin Binding Spot Test for the Detection of Carbohydrates in Torpedo AChR

To confirm the ability of the HPO-thyroglobulin to bind to lectins already coupled to AChR, increasing amounts of Torpedo receptor were spotted onto

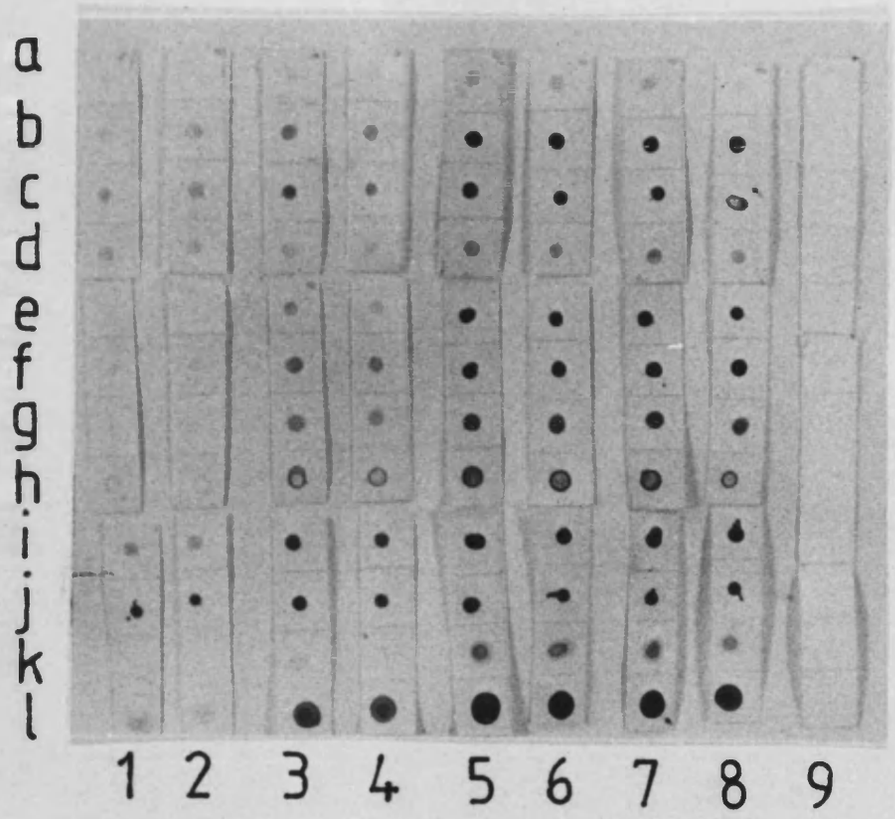
Table 4.6.2 Lectins tested for their Ability to React with HPO-Protein Conjugates

LECTIN	SUGAR SPECIFICITY
Ulex europeus	α-L-fucose
Lotus tetragonolobus	α-L-fucose
Triticum vulgaris	N-Acetyl-D-glucosamine, Sialic acid
Succinylated T.vulgaris	N-Acetyl-D-glucosamine
Limulus polyphemus	D-Glucuronic acid
Arachis hypogaea	D-Galactose
Glycine max	N-Acetyl-galactosamine, lactose
Lens culinaris	Sucrose, α-D-Glucose, N-Acetyl-D-glucosamine
Pisum sativum	D-Mannose, D-Glucose, N-Acetyl-D-glucosamine
Canavalia ensiformis	D-Mannose, D-Glucose
Phaseolus vulgaris	α-D-Galactose, N-Acetyl-D-glucosamine
Ricinus communis	D-Galactose

FIGURE 4.6.2 Spot test for testing the ability of HPO-labelled glycoprotein conjugates to bind to various lectins. Various lectins (2μg) were spotted onto nitrocellulose paper and allowed to dry prior to incubation with the conjugates (see section 3.9.4, i). The conjugates and the lectins tested are listed below. Figures in brackets represent dilution factor.

1. HPO-Ovamucoid 1:200
2. HPO-Ovamucoid 1:400
3. HPO-Thyroglobulin 1:200
4. HPO-Thyroglobulin 1:400
5. HPO-Fc$^{\gamma}$ 1:200 } unfractionated
6. HPO-Fc$^{\gamma}$ 1:400 }
7. HPO-Fc$^{\gamma}$ 1:200 } fractionated
8. HPO-Fc$^{\gamma}$ 1:400 }
9. Substrate only

a. U. europeus
b. L. tetragonolobus
c. T. vulgaris (WGA)
d. Succinylated WGA
e. L. polyphemus
f. A. hypogeae
g. G. max
h. L. culinaris
i. P. sativum
j. Con A
k. P. vulgaris
l. R. communis


a
b
c
d
e
f
g
h
i
j
k
l
1 2 3 4 5 6 7 8 9

nitrocellulose and dried. The nitrocellulose sheets were incubated with various lectins (shown below) and then with the HPO-thyroglobulin conjugate (1:400). BSA was used as a negative control.

a) Arachis hypogaea
b) Lotus tetragonolobus
c) Limulus polyphemus
d) Concanavalin A (Con A)
e) Pisum sativum (pea)
f) Lens culinaris
g) Glycine max
h) Triticum vulgaris
i) Succinylated T.vugaris

All the lectins were found to bind to Torpedo AChR. Con A bound with equal strength to all spots (1μg, 0.5μg, 0.25μg and 0.05μg). All the other lectins bound with equal strength to the 1 and 0.5μg spots and with decreasing strength to the last 2 spots. Figure 4.6.3 shows the results of the spot test.

These results confirm those produced from GLC, ie that Torpedo AChR contains fucose, mannose (glucose), galactose, N-acetylglucosamine, sialic acid and lactose.

4.6.4 Detection of the Subunit Distribution of Carbohydrates in Torpedo AChR using Lectin Binding

To locate sugar residues in the subunits, Torpedo

FIGURE 4.6.3 Spot test for the detection of lectin binding to Torpedo AChR. AChR (0.05μg - 1.0μg) was spotted onto nitrocellulose paper and allowed to dry prior to incubation with various lectins. After thorough washing, the nitrocellulose sheets were incubated with the HPO-thyroglobulin conjugate (see section 3.9.4 ii).

A) A. hypogeae

B) L. tetragonolobus

C) L. polyphemus

D) Con A

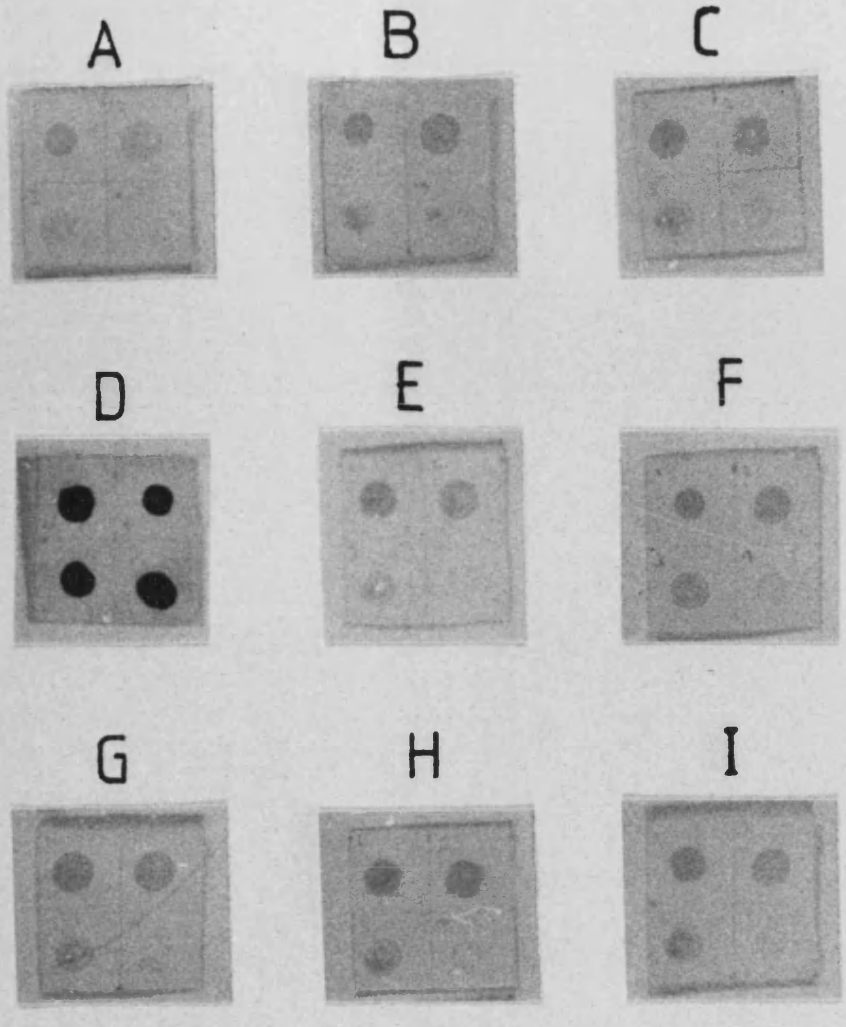
E) P. sativum

F) L. culinaris

G) G. max

H) T. vulgaris

I) Succinylated T. vulgaris



1	2
3	4

1. 1µg
2. 0.5µg
3. 0.25µg
4. 0.05µg

} AChR

AChR was transfered from gradient gels (5 - 14% SDS polyacrylamide, methods 3.9.1). The sheets of nitrocellulose were cut into strips and some strips stained with amido black to locate proteins. The ability of the subunits to bind lectins was determined as above. In some instances the receptor was first treated with either neuraminidase or galactosidase (the enzymes were removed by gel filtration as in section 3.7.2).

The results are shown in Figure 4.6.4. Standard proteins (BSA, ovalbumin, chymotrypsinogen a, ribonuclease) were used as molecular weight markers and were treated as above. In one instance, casein was used as a negative control. IgG and IgM were used as positive controls.

4.6.4.1 Subunit Distribution of Mannose/Glucose (Reaction with Con A and Pisum sativum (Pea))

All four subunits of Torpedo AChR appear to contain mannose (glucose) as shown by the binding to Con A. However, only the γ- and δ-subunits reacted with the Pea lectin.

Both γ and μ chains of IgG and IgM showed positive reactions with both Con A and Pisum sativum. Caesin was used as a negative control for P.sativum.

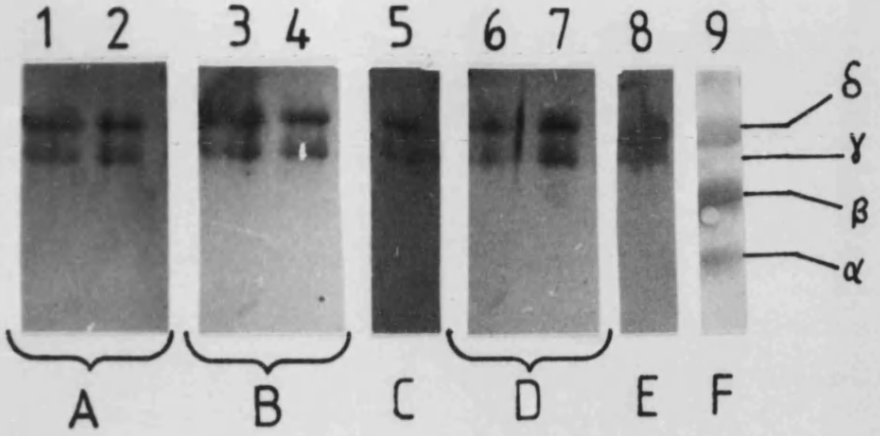


FIGURE 4.6.4 Subunit distribution of carbohydrate residues in Torpedo AChR. Following SDS-PAGE of untreated (lanes 1, 3, 5, 6, 8, 9) and neuraminidase treated (lanes 2, 4, 7) AChR, the gels were blotted onto nitrocellulose paper. Subunit distribution of sugar residues was detected following incubation with various lectins (see section 3.9.5). Lectins used are: A. T.vulgaris (N-acetylglucosamine, sialic acid), B. Succinylated T.vulgaris (N-acetylglucosamine), C. L.tetragonolobus (fucose), D. A.hypogeae (galactose), E. P.sativum (mannose, glucose), F. Con A (mannose, glucose).

4.6.4.2 Subunit Distribution of Galactose (Reaction with Arachis hypogeae lectin)

Only the γ- and δ-subunits of the AChR appeared to contain galactose as shown by the reaction with Arachis lectin. Although it might have been expected, there was no reduction of binding when galactosidase treated AChR was incubated with this lectin. Neuraminidase treatment of AChR did not result in any difference in the intensity of binding of the lectin over the untreated receptor.

Both the γ- and μ-chains of IgG and IgM reacted positively with this lectin.

4.6.4.3 Subunit Distribution of Fucose (Reaction with Lotus tetragonolobus)

Fucose appeared to be present in only two of the receptor subunits (the γ- and δ-chains) as shown by the reaction with Lotus tetragonolobus

Positive reactions to the lectin were produced with both the γ-chain and μ-chain of IgG and IgM.

4.6.4.4 Subunit Distribution of N-Acetylglucosamine and Sialic Acid (Reaction with Triticum vulgaris and Succinylated T.vulgaris

Only the γ- and δ-subunits have N-acetylglucosamine and sialic acid residues as shown by binding T.vulgaris and its succinylated derivative. Although not obvious

the photograph, the neuraminidase treated AChR gave a less strong reaction than the untreated AChR when incubated with Triticum lectin. There was no difference in the intensity of binding of the treated and untreated AChR to the succinylated Triticum lectin.

Positive reactions to Triticum lectin and the succinylated derivative were produced by both the γ- and δ-chains of IgG and IgM.

4.7 DETECTION OF ACETYLCHOLINE RECEPTOR CARBOHYDRATE MOIETY USING AN ENZYME-LINKED LECTIN BINDING ASSAY (ELBA)

4.7.1 Detection of Mannose (Glucose) in IgM using the ELBA

An assay was developed (based on the enzyme-linked immunosorbant assay) to enable the detection of carbohydrates in glycoproteins immobilised on plastic cuvettes. Cuvettes were coated with IgG as described in section 3.10 and then incubated with Con A (specific for mannose/glucose) (0 - 14μg/ml). Horseradish peroxidase was used to detect lectin binding. Gelatin, used as a negative control, was treated as for Con A. The presence of mannose (glucose) in IgM was confirmed by its binding to Con A. Figure 4.7.1a shows the results of these investigations.

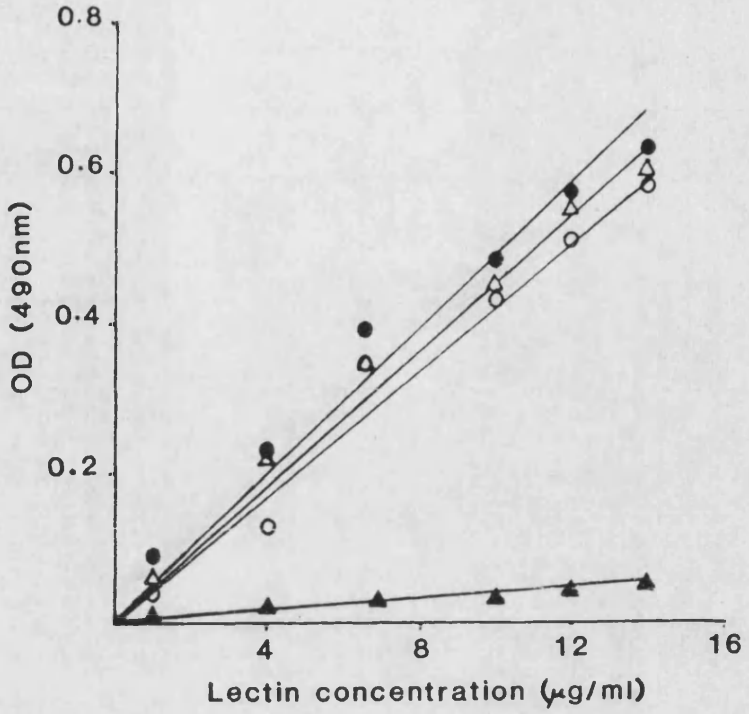
Binding of a fixed amount of Con A (1μg/ml) to IgM was inhibited by coincubation with increasing amounts of

Figures 4.7.1

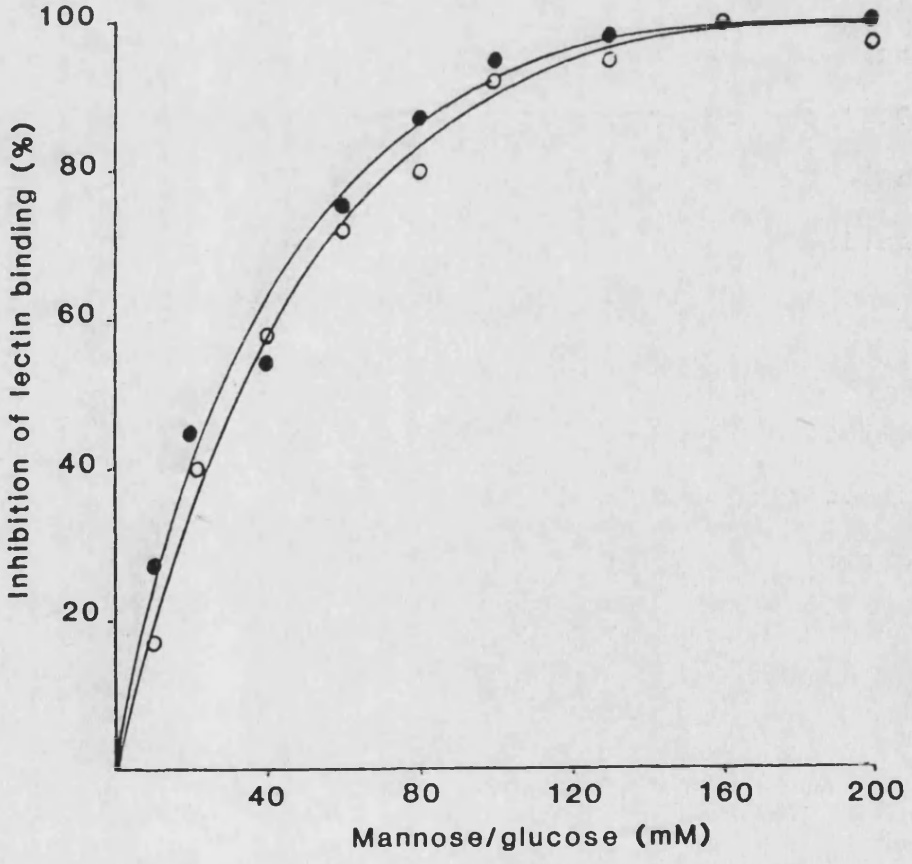
a) Con A binding to glycoprotein coated plastic cuvettes. The cuvettes were incubated with increasing amounts of Con A for 2h at 37°C or overnight at 20°C. The cuvettes were repeatedly washed with PBS-Tween and incubated as for Con A with a solution of horseradish peroxidase (1μg/ml) in PBS and then washed as before. Colour was developed by adding 0.04% (w/v) o-phenylenediamine in phosphate-citrate buffer, pH 5.0, containing 0.012% (v/v) hydrogen peroxide. The reaction was stopped by the addition of 60% H_2SO_4 (50μl) and the colour read at 490nm. (●) Untreated AChR, (Δ) neuraminadase treated AChR, (O) IgM, (▲) gelatin.

b) Inhibition of Con A binding to glycoprotein coated cuvettes. The assay was carried out as above but using a fixed amount of Con A (1μg/ml) and in the presence of increasing concentrations of mannose/glucose (0-200mM in the final solution). (●) Untreated AChR, (O) IgM.

a)


0.8
0.6
0.4
0.2
OD (490nm)
4
8
12
16
Lectin concentration (μg/ml)

b)


100
80
60
40
20
Inhibition of lectin binding (%)
40
80
120
160
200
Mannose/glucose (mM)

mannose/glucose (0 - 200mM in the final solution) and was shown to exhibit 100% inhibition at approximately 160mM mannose. See Figure 4.7.1b.

4.7.2 Detection of Mannose (Glucose) in Torpedo AChR using the ELBA

The presence of mannose (glucose) in the acetylcholine receptor was demonstrated by the enzyme-linked lectin binding assay. The receptor was coated onto cuvettes and the assay carried out as described above. Neuraminidase treated receptor was also included in the assay. See Figure 4.7.1a.

Binding of both treated and untreated AChR to a fixed amount (1μg/ml) of Con A could be inhibited by coincubation with increasing amounts of mannose/glucose (0 - 200mM in the final solution). Total inhibition of binding was achieved with concentrations of approximately 145mM and 140mM mannose/glucose (for the untreated and treated AChR respectively). See Figures 4.7.1b.

4.7.3 Detection of Fucose in IgM using the ELBA

The presence of fucose in IgM was detected by the ELBA. IgM was coated onto cuvettes, incubated with increasing amounts of Lotus tetragonolobus lectin (0 - 14μg/ml) and the assay carried out as described previously. As horseradish peroxidase does not bind with

the Lotus lectin used for the detection of fucose residues, Lotus lectin commercially coupled to HPO was used. As with Con A, gelatin was used as a negative control. Figure 4.7.2a shows that the assay can be successfully used for the detection of fucose residues in immobilised IgM.

Binding of IgM to a fixed amount of Lotus lectin (1μg/ml) could be inhibited by coincubation with fucose (0 - 200mM in the final solution). Total inhibition was achieved at a fucose concentration of 115mM (Figure 4.7.2b).

4.7.4 Detection of Fucose in Torpedo AChR using the ELBA

Cuvettes were coated with acetylcholine receptor as described previously and incubated with HPO-Lotus lectin. The AChR produced a positive reaction when incubated with the lectin as did neuraminidase treated receptor (Figure 4.7.2a).

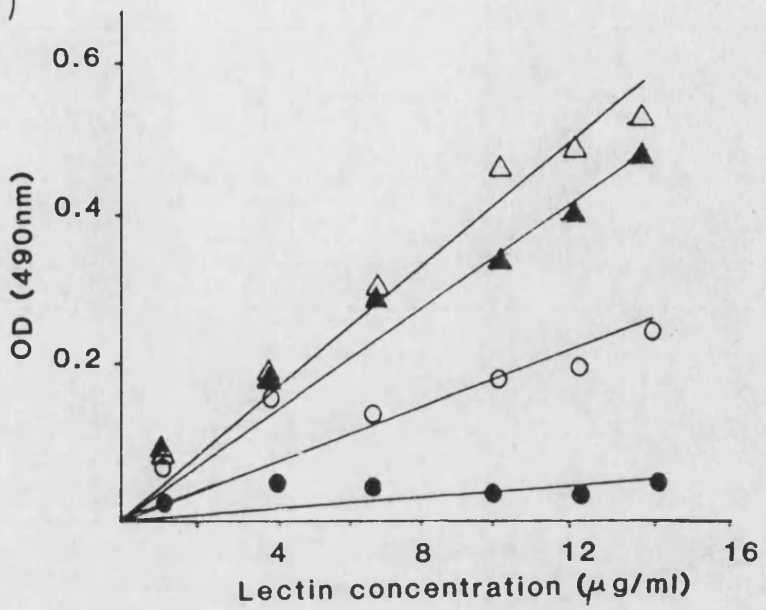
Binding of the AChR to a fixed amount of HPO-Lotus lectin (1μg/ml) could be inhibited by coincubation with fucose (0 -200mM in the final solution). Complete inhibition of binding was achieved at a concentration of 125mM fucose (see Figure 4.7.2b).

Figures 4.7.2

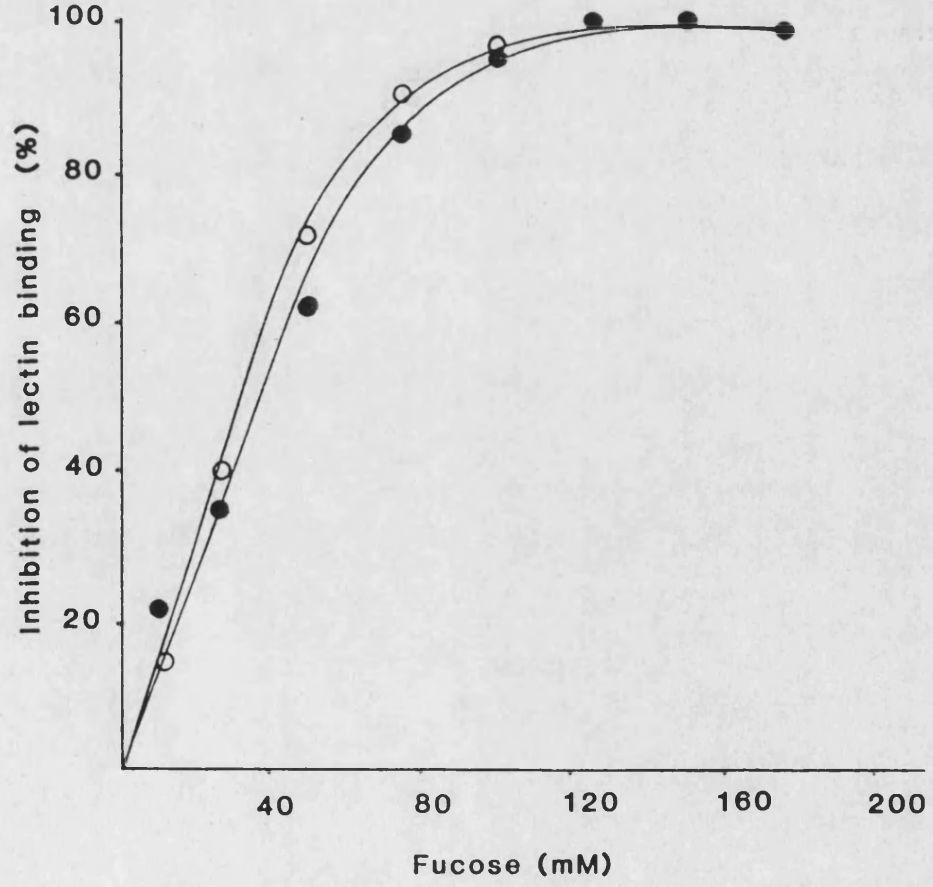
a) L.tetragonolobus lectin binding to glycoprotein coated plastic cuvettes. The cuvettes were incubated with increasing amounts of HPO-Lotus lectin for 2h at 37°C or overnight at 20°C. The cuvettes were repeatedly washed with PBS-Tween and the colour developed by adding 0.04% (w/v) o-phenylenediamine in phosphate-citrate buffer, pH 5.0, containing 0.012% (v/v) hydrogen peroxide. The reaction was stopped by the addition of 60% H_2SO_4 (50µl) and the colour read at 490nm. (▲) Untreated AChR, (△) neuraminadase treated AChR, (o) IgM, (●) gelatin.

b) Inhibition of lectin binding to glycoprotein coated cuvettes. The assay was carried out as above but using a fixed amount of lectin (1µg/ml) and in the presence of increasing concentrations of fucose (0-200mM in the final solution). (o) Untreated AChR, (●) IgM.

a)


0.6
0.4
0.2
OD (490nm)
4
8
12
16
Lectin concentration (μg/ml)

b)


100
80
60
40
20
Inhibition of lectin binding (%)
40
80
120
160
200
Fucose (mM)

4.7.5 Inhibition of HPO-Lotus Lectin Binding to Acetylcholine Receptor with Glycoproteins

Binding of monosaccharides to lectins is highly specific, however, lectin binding is much stronger when the sugar is presented to the lectin in a chain such as an oligosaccharide or glycoprotein. This can be demonstrated by inhibiting lectin binding to AChR by using AChR or IgM. Fetuin was used as a negative control. The results of this experiment are shown in Figure 4.7.3a and b. No inhibition of lectin binding to acetylcholine receptor could be demonstrated using fetuin. Inhibition of binding by AChR was demonstrated. However, only 65% of the total binding could be inhibited using acetylcholine receptor. Inhibition of lectin binding to AChR was also evident by using IgM and as expected the inhibition was less than that achieved with acetylcholine receptor.

4.8 DETECTION OF CARBOHYDRATE RESIDUES IN TORPEDO ACETLYCHOLINE RECEPTOR BY INHIBITION OF LECTIN INDUCED HAEMAGGLUTINATION

The presence of various sugar residues in Torpedo AChR could easily be detected by the ability of the receptor to inhibit lectin-induced haemagglutination. With the exception of the U.europeus lectin, all lectin-induced agglutination was inhibited to some degree by the Torpedo receptor (Table 4.8.1).

Figures 4.7.3a, b. Inhibition of L.tetragonolobus lectin binding to AChR coated plastic cuvettes. The cuvettes were incubated with a fixed amount (1μg/ml) of HPO-Lotus lectin for 2h at 37°C or overnight at 20°C in the presence of increasing concentrations of either AChR or IgM. The cuvettes were repeatedly washed with PBS-Tween and the colour developed by adding 0.04% (w/v) o-phenylenediamine in phosphate-citrate buffer, pH 5.0, containing 0.012% (v/v) hydrogen peroxide. The reaction was stopped by the addition of 60% H_2SO_4 (50μl) and the colour read at 490nm. (●) AChR, (○) IgM.

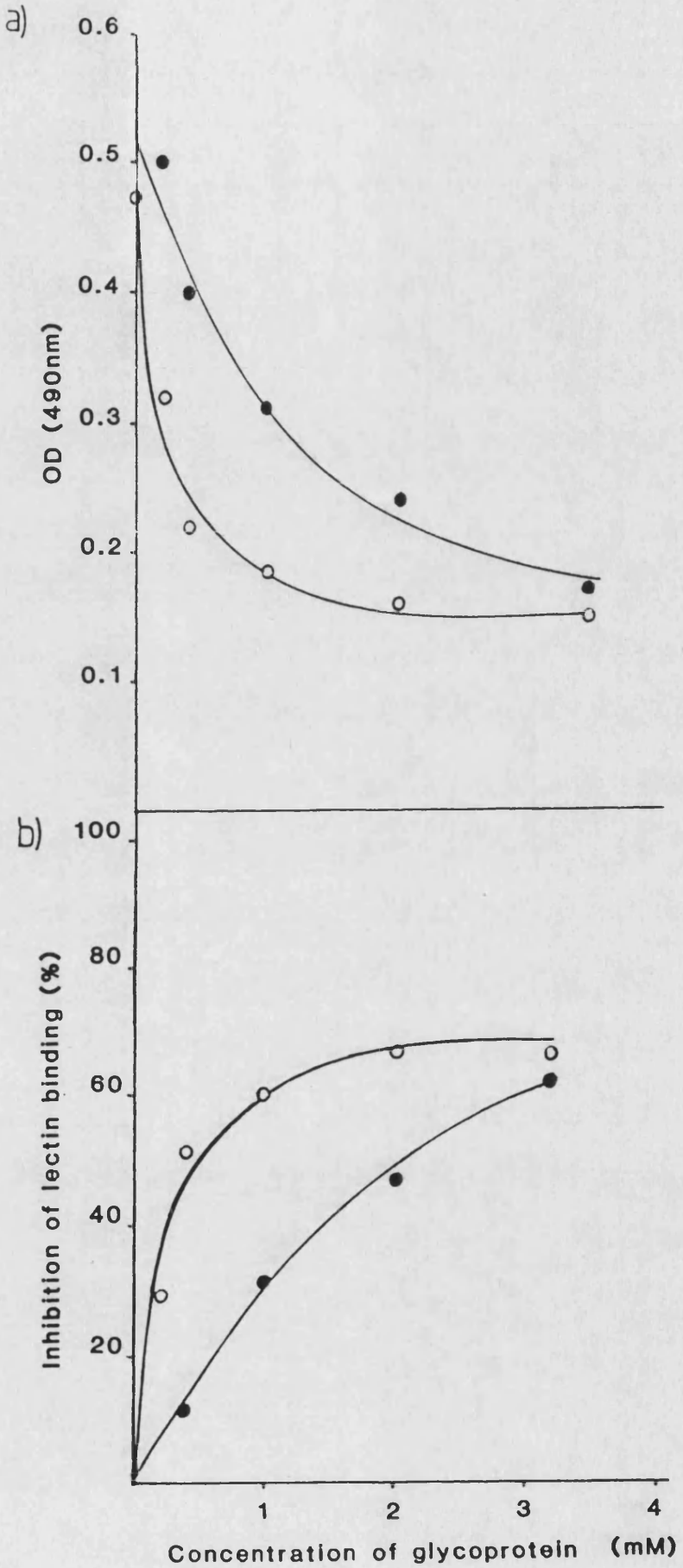

a)
0.6
0.5
0.4
0.3
0.2
0.1
OD (490nm)
b)
100
80
60
40
20
Inhibition of lectin binding (%)
1
2
3
4
Concentration of glycoprotein (mM)

Table 4.8.1 Inhibition of Lectin Haemagglutination by Torpedo AChR

LECTIN	pmol AChR/WELL										
	135	67	33	16	8	4	2	1	0.5	0.25	Control
L.culinaris	-	-	-	±	+	+	+	+	+	+	-
T.vulgaris	-	-	-	±	+	+	+	+	+	+	-
P.sativum	-	-	-	±	+	+	+	+	+	+	-
G.max	-	-	-	-	-	-	±	+	+	+	-
L.tetragonolobus	-	-	-	±	+	+	+	+	+	+	-
U.europeus	+	+	+	+	+	+	+	+	+	+	-
C.ensiformis	-	-	-	-	-	±	+	+	+	+	-

+ - Agglutination
- - No agglutination
± - End point
Control - Red blood cells only

4.9 PASSIVE HAEMAGGLUTINATION TEST

4.9.1 Effect of Triton X-100 on Sheep Red Blood Cells

As AChR is solubilised by Triton X-100 extraction, the effect of this non-ionic detergent on SRBC was initially investigated. Serial dilutions of 1% (v/v) Triton X-100 were carried out in 0.05ml volumes of saline in microtitre plates. 0.05ml of a 1% (v/v) SRBC suspension in saline was added to each well and cell lysis observed after 2h incubation at 20°C and 37°C. The detergent caused lysis at concentrations 0.1% (v/v). The purified receptor preparations which contained 0.1% (v/v) Triton X-100 were dialysed for 24h against saline to remove phosphate ions which inhibit $CrCl_3$ coupling (Goding, 1976) and also to remove excess detergent monomers. Dialysis does not remove protein-bound and protein-free detergent micelles (Furth, 1980) and, therefore, the receptor remains soluble and no aggregation of the protein is observed. Early attempts at removing the detergent by hydrophobic adsorption with Biobeads, Sm2 (Holloway, 1973) resulted in the aggregation and precipitation of the receptor and loss of αBGT binding activity.

4.9.2 α-Bungarotoxin-binding to AChR-coated SRBC

AChR is usually quantitated and characterised in

terms of αBGT binding and, therefore, the ability of coated cells to bind this neurotoxin was determined (see section 3.12.3). Figure 4.9.1a shows that the bound receptor still retained toxin binding activity. This was specific (displaceable by the cholinergic ligand benzoquinonium chloride). Haemoglobin-coated RBC showed negligible binding which was not displaceable by benzoquinonium chloride. The Scatchard plot (Figure 4.9.1b) gave an apparent Kd value of 55nM and a Bmax of 0.69 moles.

4.9.3 Effect of the Amount of Chromic Chloride Used

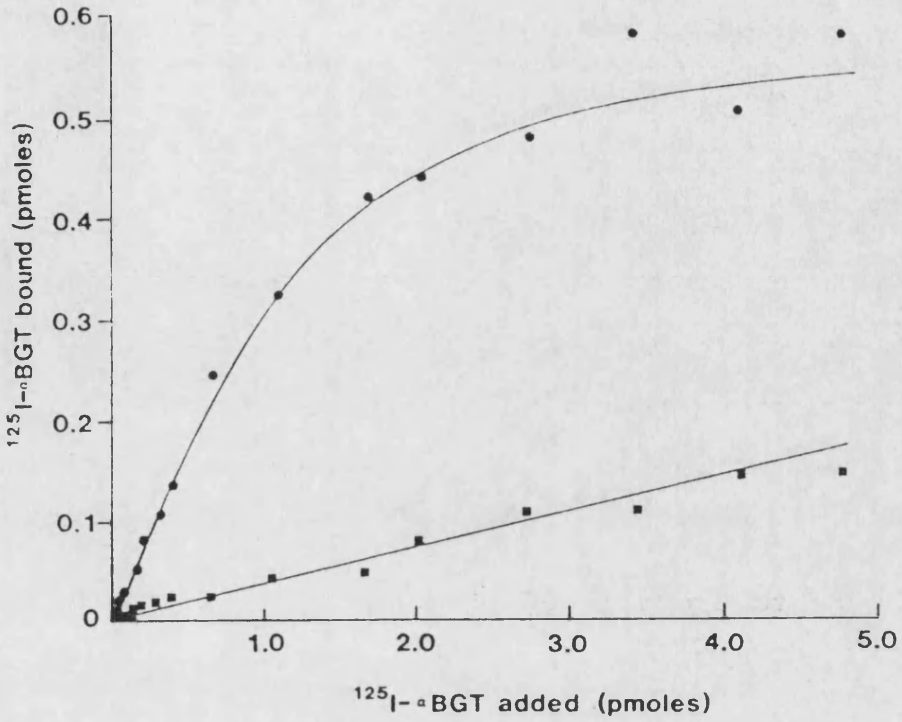
The amount of $CrCl_3$ used influences both the uptake of protein and the agglutinability of the coated red cells. AChR uptake was measured in terms of ^{125}I-αBGT binding, and cell agglutination was examined using rabbit anti-AChR antiserum. Table 4.9.1 shows that the uptake of AChR increased with increasing amounts of $CrCl_3$ solution used. However, the strongest agglutination was obtained with cells coated using 0.35ml of $CrCl_3$ solution. Larger volumes of $CrCl_3$ solution yielded cells which agglutinated spontaneously. Therefore, all subsequent coating was carried out with 0.35ml of $CrCl_3$ solution.

FIGURE 4.9.1. ^{125}I-αBGT binding to AChR-coated red cells.

(a) Increasing concentrations of ^{125}I-αBGT were incubated with 20μl of coated cell suspension as described in the methods section. Non-specific binding (■) was obtained from binding in the presence of benzoquinonium chloride. Specific binding (●) was obtained after subtraction of non-specific from total binding. Specific binding of ^{125}I-αBGT to haemaglobin-coated cells was negligible (not shown).

(b) Scatchard plot of the data represented in (a).

(a)


0.6
0.5
0.4
0.3
0.2
0.1
0
1.0
2.0
3.0
4.0
5.0
^{125}I-$^{\alpha}$BGT bound (pmoles)
^{125}I-$^{\alpha}$BGT added (pmoles)

(b)

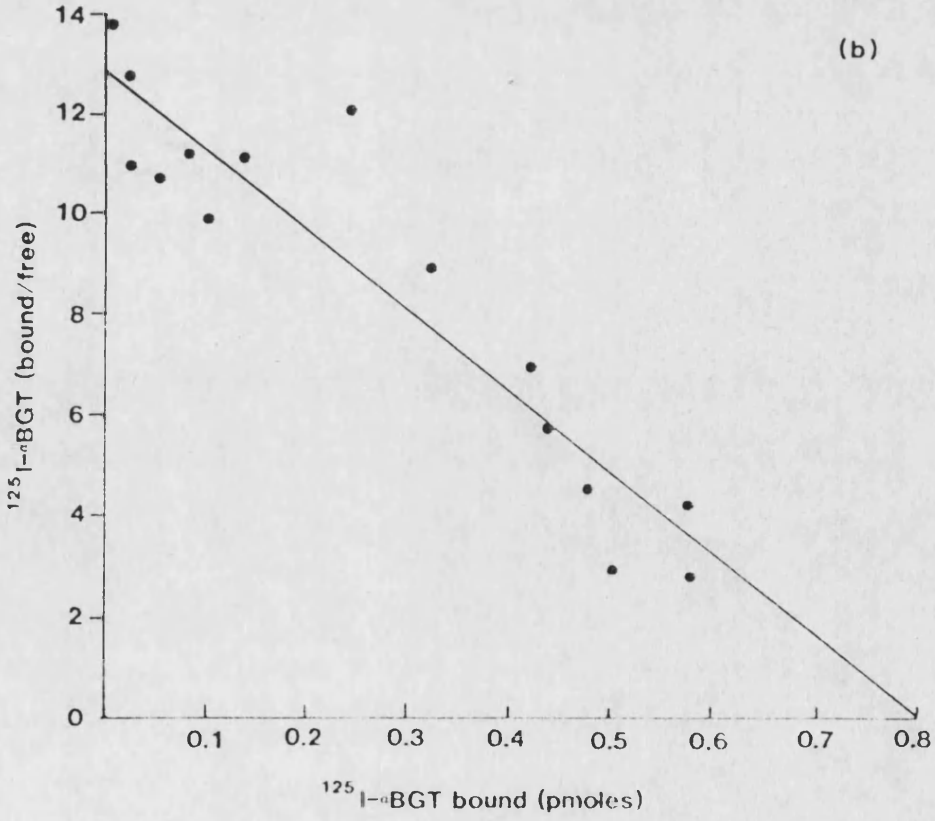

14
12
10
8
6
4
2
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
^{125}I-$^{\alpha}$BGT (bound/free)
^{125}I-$^{\alpha}$BGT bound (pmoles)

Table 4.9.1 Effect of Volume of $CrCl_3$ Solution Used For Coupling AChR To SRBC

VOL OF $CrCl_3$ SOLUTION USED (ml)	STRENGTH OF TEST[a] AGGLUTINATION	CONTOL[b]	^{125}I-αBGT[c] BINDING pmoles/20μl of 1% SRBC
0	-	neg	N.D
0.1	-	=	0.035
0.2	±	=	0.066
0.25	+	=	0.112
0.30	++	=	0.197
0.35	+++	=	0.530
0.40	++	+	0.722

a) Haemagglutination performed with rabbit anti-Torpedo AChR antiserum.

b) Haemagglutination performed with normal rabbit serum.

c) Toxin binding was carried out using excess ^{125}I-αBGT (4.5 pmole)

N.D = not detected.

4.9.4 Haemagglutination of AChR-coated red cells

Several polyclonal and monoclonal antisera to AChR were titrated against AChR-coated red cells. The results are summarised in Table 4.9.2 and an example of the agglutination pattern obtained is shown in Figure 4.9.2. With polyclonal antisera the agglutination titres correlated well with titres measured by radioimmunoassay. Immunological cross-reactivity between *Torpedo* AChR and receptor from other species is also detected by the agglutination test. Complement-mediated lytic activity was shown by two antisera only.

Agglutination of receptor coated red cells with monoclonal antibodies (mAb) was different. First, end-points were achieved with relatively higher amounts of antibodies. Second, there was no correlation between the agglutination and radioimmunoassay titres.

Table 4.9.2 Titration of Anti-AChR Antisera

ANTISERUM		ANTI-TORPEDO AChR[a] ANTIBODY TITRE (RADIOIMMUNOASSAY) (nM)
Normal rabbit serum		N.D
Rabbit anti Torpedo AChR		14,500
Normal sheep serum		N.D
Sheep anti Torpedo AChR		6400
Rabbit anti-rat AChR		1517 (42,000)
Rabbit anti-foetal calf AChR	(1)	17.5 (320)
	(2)	5.5 (700)
Non-immune mouse ascitic fluid		N.D
Mouse monoclonal anti-Torpedo AChR		
A3		2350
B11		170
C2		103
C7		143

a) Values in brackets are antibody titres i.e., rat AChR and foetal calf AChR.

b) Titration of anti-Torpedo AChR antisera agglutination in the first well only.

N.D = not detected.

with AChR-Coated Red Cells

HAEMAGGLUTINATION TITRE	COMPLEMENT-MEDIATED LYSIS TITRE
1:2	
1:655,360	1:128
-	
1:163,840	
1:40,960	
1:256	1:32
1:8	
1:2	
1:1024	
1:16	
1:16	
1:1280	

measured using the primary antigen

against haemoglobin coated SRBC showed

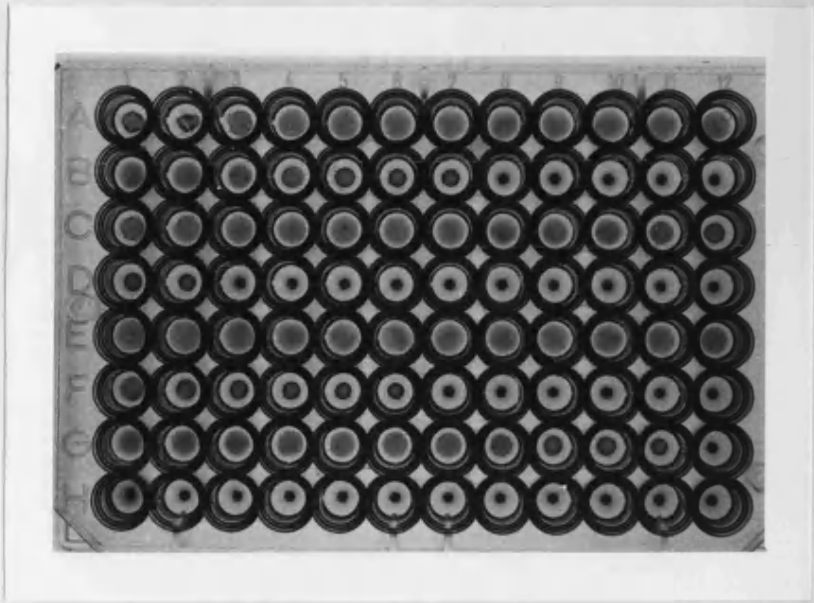


FIGURE 4.9.2 Haemagglutination patterns of AChR-coated RBC.

Serial dilutions of antisera were made in two rows and agglutination performed with AChR-SRBC.

Rows A and B, rabbit anti-Torpedo AChR antiserum (well 1 dilution 1:20). End-point = 1:655,360 dilution.

Rows C and D, rabbit anti-rat AChR antiserum (well 1 dilution 1:20). End-point = 1:40,960 dilution.

Row E and F, sheep anti-Torpedo (well 1 dilution 1:20). End-point = 1:63,840 dilution.

Rows G and H, monoclonal antibody (A3) (well 1 dilution

DISCUSSION

5.1 The Preparation and Characterisation of Radiolabelled α-Bungarotoxin

For certain parts of the work covered by this research project it was essential to obtain an accurate assessment of the concentration of nicotinic acetylcholine receptor in a preparation. The accuracy of this assessment is, in part, dependent on the method of determination employed, ie, the toxin binding assay. The assay involves the incubation of acetylcholine receptors with radiolabelled α-bungarotoxin and subsequent separation of the receptor-toxin complex from free labelled toxin. Calculation of the amount of receptor bound to toxin is, therefore, dependent on having an accurate knowledge of the concentration of labelled toxin added to the incubation mixture and on having a reliable means of separating bound from free radiolabelled toxin.

Purification of αBGT from venom and repurification of commercially purified αBGT was carried out in order to ensure the purity of commercially available αBGT. Repurification of αBGT revealed a small peak consisting of approximately 5% of the total protein. This could lead to an overestimation of the actual concentration of αBGT present in the binding assay. Hence, there may have been an underestimation of the amount of receptor present in any particular preparation.

Iodination frequently alters the binding activity of proteins and it has been reported (Potter, 1974) that radiolabelling may modify the binding of the iodinated toxin towards the acetylcholine receptor. Thus a brief examination of the method of iodinating αBGT was carried out. Three methods were investigated: these used Chloramine T (Hunter, 1978) iodine monochloride (Vogel et al., 1972) and iodine monochloride by a modified procedure (Doran and Spar, 1980). The biological activities of the iodinated preparations were compared when the chloramine T and iodine monochloride (Vogel et al., 1972) methods were found to produce comparable results, whereas yields from the modified iodine monochloride method were consistently very low. This indicated, at least for the purposes of this study, that the Chloramine T method of iodination caused no more radiological damage to the toxin than did the other methods.

James et al. (1980) recommended that the products of the iodination of αBGT should be fractionated to remove $^{125}I_2$-αBGT, as this derivative has a modified reactivity towards the receptor. Results from the present study, however, (Table 4.1.3) show that although there was a small increase in specific binding of mono-iodinated αBGT compared with that of unfractionated ^{125}I-αBGT (average 10%), this increase did not justify fractionation of radiolabelled α-toxin derivatives on a regular basis for accurate quantitation of Torpedo AChR binding sites.

Very little difference was found between the immunological activities of commercial toxin and toxin which had been purified from venom.

To assess the accuracy of the gel filtration method for determination of the concentration of iodinated αBGT alternative competition studies were carried out. These were found to agree with results from gel filtration.

In conclusion, it was decided that for the purposes of quantitating Torpedo acetylcholine receptor binding sites, iodination of commercially available α-bungarotoxin by the Chloramine T method and estimation of the toxin concentration by the gel filtration method was adequate. It was also considered unneccessary to fractionate the iodinated derivatives.

5.2 Purification of AChR

5.2.1 Modifications of the Method of Purification of Torpedo Acetylcholine Receptor

Modifications to the initially used method of purification (Lindstrom, 1976) of the AChR from the electric organs were considered necessary for two main reasons. These were: firstly, to improve overall yields of the purified glycoprotein, and secondly, to increase the specific toxin-binding activity of the receptor.

Four modifications (described in Methods 3.3.2) to the basic purification procedure were considered in order to improve the yield of AChR. These resulted in increase in the total amount of protein and toxin binding sites purified. Modifications included, firstly, the homogenisation of the frozen tissue in salt solution, rather than phosphate buffer. The salt may have improved yields by early removal of proteins ionically-bound to membranes. Secondly, high speed centrifugation of the homogenate resulted in a larger proportion of the membrane fragments, and hence AChR, being pelleted. Thirdly, the subsequent resuspension of these membrane fragments by the use of a Polytron probably disrupted the membranes, so rendering the receptor more accessible for solubilisation. Finally, although the modifications described above undoubtably contributed to the yield, and possibly purity, of the acetylcholine receptor, the major

contributing factor to the improved yield (Table 4.3.1) of the AChR is probably the use of 2% Triton X100 for solubilisation. Benzoquinonium chloride was used, in several instances, to elute the acetylcholine receptor from the α-Naja affinity column. The overall yield of AChR following desorption by benzoquinonium was consistently lower than that obtained following carbachol desorption. A similar result has been shown by Eldefrawi and Eldefrawi (1973) who found that benzoquinonium-desorbed receptor contained only 80% of the carbamylcholine-desorbed receptor. Furthermore, after desorption by benzoquinonium, treatment of the affinity column with 1M carbamylcholine resulted in the recovery of an additional 15% of the protein.

The specific binding activity of the nicotinic acetylcholine receptor from Torpedo has been reported to be in the range of 1.7 - 9.0 nmol/mg protein (see Table 5.1). In this study, specific activities of AChR purified by the method of Lindstrom (1976) were in the range of 0.6 - 6.1 nmol/mg (mean 3.0 nmol/mg) which appear low compared with the literature. However, one of the major complications encountered when comparing specific activities quoted by various workers is caused by the use of different assay methods. Mehraban *et al* (1982), used a similar purification procedure to that described here but with the substitution of the DEAE column with a lectin (*Lens culinaris*) column. They reported values of 4 - 6 nmol toxin bound/mg protein, which values are comparable to those reported in this

Table 5.1 Specific Activities of AChR from Fish Electric Organs

SOURCE	SPECIFIC ACTIVITY (nmol neurotoxin/ mg protein)	REFERENCES
Electrophorus electricus	4.5	Bisecker (1973)
	5.4	Hucho & Changeux (1973)
	8	Karlin & Cowburn (1973)
		Karlin et al (1976)
	11	Klett et al (1973)
	5-6.5	Chang (1974)
		Penn et al (1976)
	6.7	Meunier et al (1974)
		Sobel et al (1977)
	7.5	Lindstrom & Patrick (1974)
	3.6	Patrick et al (1975)
	6.3	Schmidt & Raftery (1973)
T.californica	8	Weill et al (1974)
	10	Eldefrawi et al (1975b)
	12	Chang & Brock (1977)
	8	Froehner et al (1977a)
		Froehner & Rafto (1979)
	10.6	Barfeld & Fuchs (1979)
	8-9	VanOlen et al (1979)
	9	Ruchel et al (1981)
T.marmorata	9	Potter (1973)
	1.7	Heilbronn & Mattsson (1974)
	5	Gordon et al (1974)
	8	Eldefrawi et al (1975a)
	7-9	Sobel et al (1977, 1978)
	9.6	Vandlen et al (1979)
	9	Ruchel et al (1981)
	4-6	Mehraban et al (1982)
T.ocellata	10-12	Rubasmen et al (1978)
T.nobliana	12.5	Ong & Brady (1974)
Narcine entemedor	2.7	Schmidt & Raftery (1972)
N.brasilienis	9	Chang et al (1977)
Narke japonica	2.2	Ishikawa (1980)

thesis. Modifications to the purification procedure had little appreciable effect on specific binding activities.

The relatively low specific activity of receptor preparations found here compared to those reported by other workers may be as a result of the iodinated toxin preparations used to assay the AChR. In a number of reports (Ruchel et al., 1981, Mehraban et al., 1980, Sobel et al., 1977, Mattsson and Heilbronn, 1974), tritiated αBGT has been used to assay toxin binding sites and this radiolabelled antagonist has been shown to have a biological activity in the region of 100% (Lo et al., 1981).

My specific activities (0.6 - 10.7 nmol/mg) in fact, compare very well with those previously reported by this laboratory (3.5 - 4.4 nmol/mg).

5.2.2 Purification of Torpedo acetylcholine receptor on an Anti-(Torpedo AChR) Monoclonal Antibody-Affinity Column

Recoveries (7.0 ± 1.0 mg/Kg organ) of Torpedo acetylcholine receptor from electroplaque tissue obtained by affinity chromatography on anti-(Torpedo AChR) monoclonal antibodies were considerable lower than those reported by Lennon et al., (1980) (62 - 95 mg/Kg of organ). Lennon et al., (1980) also reported specific activities of 4.9 ± 0.35 pmol/μg which are approximately 2.5 times higher than those found here (1.9 ± 0.3 pmol/μg). The lower specific activity obtained in the present studies

may arise from a delay in neutralising the receptor solution eluted from the affinity column. It has been shown that exposure of AChR to pH values greater than 10 results in a large reduction in α-bungarotoxin binding sites (Lennon et al., 1980) and while the pH of the elution buffer, in this case, was only 10, some loss of binding sites may have occured.

Differences between yields and specific activities of the receptor prepared here and that of Lennon et al., (1980) may also be a function of the antibody population. Antibodies are highly specific and the population of antibodies used here may be less specific than those of other workers or they may bind so tightly that displacement of them from the affinity column may require extreme conditions.

A more extensive investigation of the monoclonal antibody population would need to be carried out before accurate conclusions can be drawn concerning purification of the receptor by this method.

5.3 Chemical Analysis of AChR Carbohydrate Moiety

5.3.1 Sialic Acid Content of Torpedo Acetylcholine Receptor

The presence of sialic acid in the carbohydrate moeity of the Torpedo acetylcholine receptor has been reported (Vandlen et al., 1979, Lindstrom et al., 1979, Bersinger et al., 1982). Sialic acid is a common constituent of a number of soluble glycoproteins (e.g. mucins, immunoglobulins, fetuin, ribonuclease b, thyroglobulin) and of membrane glycoproteins (e.g. Band 3 glycoprotein of erythrocyte membranes). Also, exogenously administered desialylated serum glycoproteins have been shown to be rapidly cleared from the circulation (Ashwell and Morell, 1974), thus implicating the sialic acid as a regulatory determinant for the catabolism of glycoproteins. It is, therefore, not unreasonable to have expected to find sialic acid as a constituent of the saccharide structure of the AChR.

The amount and subunit distribution of sialic acid in the AChR has received only rudimentary investigation. Vandlen et al., (1979) reported trace amounts of sialic acid on all subunits of the receptor, whereas Lindstrom et al., (1979) found substantial amounts on only the γ- and δ-subunits (9.6nmol/mg and 10nmol/mg for the γ- and δ-subunits respectively). While, at this point, no subunit distribution of sialic acid can be ascertained a

figure of 8 ± 0.16 nmol sialic acid per mg protein is identical to that determined by Bersinger et al., (1982).

The slight variablity of the sialic acid content of the acetylcholine receptor may be due to peripheral heterogeneity (Marshall and Neuberger, 1970).

Sialic acid was successfully cleaved off the acetylcholine receptor by neuraminidase treatment (50 - 100% by weight of total sialic acid present). The neuraminidase from Clostridium perfringens exhibits maximal activity towards the α-(2→3) linkage and approximately 50% of this activity towards the α-(2→6) and α-(2→8) glycosidic linkages (for review see Schauer, 1982). The failure of the neuraminidase (in some instances) to remove 100% of the sialic acid may be as a result of heterogeneity in that not all the glycosidic bonds are α-(2→3). Also, neuraminidase from C. perfringens has 1/5 the activity towards N-glycolyl-neuraminic acid compared with that towards N-acetyl-neuraminic acid.

5.3.2 Quantitative Analysis using Gas-liquid Chromotography of the Monosaccharide Residues of Intact Torpedo Acetylcholine Receptor

Torpedo acetylcholine receptor has been reported to contain the monosaccharides mannose, glucose, galactose and N-acetylglucosamine (Vandlen et al., 1979, Lindstrom et al., 1979).

Three colorimetric methods have been used to assay

the total sugar content of the acetylcholine receptor, these include the Anthrone method (Colowick and Kaplan, 1957), the phenol-sulphuric acid method (Dubois et al., 1956) as used by Vandlen et al., (1979) and the cysteine-sulphuric acid method of Dische and Danilcheneko (1967). All three methods have the disadvantage that they do not produce a reaction with amino sugars and the reaction can be affected by such substances as tryptophan and so were rejected.

Analyses of the acetylcholine receptor by using gas-liquid chromatography have produced values for the total sugar content of the receptor of 38 ± 12μg/mg (Mattsson and Heilbronn, 1975) and 40 - 70μg/mg protein (Lindstrom et al., 1979), while that reported here was around 70μg/mg. The lower values reported by other investigators probably arise from the different derivatisation methods employed for preparing samples. GLC analysis of the receptor showed fucose, mannose, glucose, galactose, N-acetyl-glucosamine (47, 123, 40, 21 and 42 nmol/mg respectively) which compare extremely well with values reported by Lindstrom et al., (1979), who, from a single set of GLC analyses, determined the sugars present to be mannose, glucose, galactose and N-acetyl-glucosamine (115, 53, 22 and 54 nmol/mg respectively), the presence of fucose was not reported.

Vandlen et al., (1979) assayed the sugar content of the acetylcholine receptor from T.californica by using the phenol-sulphuric acid method and obtained a value of 194 nmol of carbohydrate per mg protein. While this

figure is low compared to that determined here from GLC analysis (290 ± 25 nmol/mg), if allowances are made for amino sugars (which would not have been detected by the colorimetric method) a value of 278 nmol/mg (84 nmol from N-acetylglucosamine) is obtained. The value thus obtained compares well with that shown here.

The presence of fucose in the acetylcholine receptor from T.marmorata has not been found by any other investigators, however, it has previously been detected in this laboratory and was confirmed by lectin binding studies (see sections 4.6, 4.7 and 4.8).

The presence of glucose in the acetylcholine receptor was debatable as it was not detected in 40% of the preparations examined. Glucose is only very rarely found in glycoproteins and as no lectin has yet been discovered that is specific for glucose it has not been possible to confirm its presence by lectin-binding studies. Glucose may be present as a contaminant from the agarose column used during purification and this may substantially alter the small amounts of sugars detected (Briley and Changeux, 1977). However, extensive dialysis of preparations was carried out after the final stage of purification and, therefore, any free monosaccharides would have been removed. Also, no trace of any sugar was detected in samples of buffer eluted from Sephadex, Sephacryl or Ulragel ACA columns. It is, moreover, notoriously difficult to guard against traces of cellulose fibres in small scale analytical samples. The extreme variability of the glucose content of the

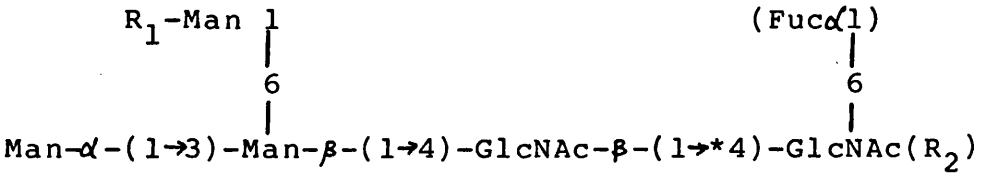
receptor could conceivably result from structural heterogeneity. Differences in the structures of glycoproteins can manifest themselves in a number of ways. These include heterogeneity of nonreducing, terminal sugar residues (peripheral heterogeneity) and, also more complex variation in the structure of the carbohydrate moieties of glycoproteins. An example of peripheral heterogeneity can be found in collagen, the carbohydrate moiety of which occurs, in part, as 2-O-α-D-glucopyranosyl-O-β-D-galactopyranosyl and, in part, as O-β-D-galactopyranosyl groups, ie both with and without the terminal D-glucopyranosyl groups (Marshall and Neuberger, 1970). More complex structural heterogeneity has been shown in the glycoprotein ovalbumin from egg white (Huang et al., 1970). The carbohydrate structure of ovalbumin was only confirmed after considerable effort was put into eliminating such variables as genetic variants, ages of egg and possible presence of glycosidase activity.

5.3.3 Effect of Enzyme Treatment on the Sugar Content of Torpedo AChR

Enzyme treatment of Torpedo acetylcholine receptor was approached in two ways. The first was the treatment of the receptor with mixed glycosidases from Trichomonas foetus. The glycosidase extract has been found to contain fucosidase, mannosidase, galactosidase and hexosaminidase activity (Westwood et al., 1976, Watkins, 1966). Treatment with these enzymes produced a reduction of the total amount of sugars of up to 60%. From a single incubation of the enzymes with acetylcholine receptor it would not be expected that 100% of the sugars would be removed as it is common for an enzyme to be inhibited by the end product of the reaction (eg α-mannosidase). Failure of the enzymes to completely remove all the monosaccharide residues may also be due to the inability of the enzyme to hydrolyse the remaining glycosidic bonds, eg α-L-fucosidase from T.foetus will only cleave α-L-fuc-(1$\rightarrow$3 or 4)-GlcNAc, and thus fucose linked 1$\rightarrow$6 would be unaffected by this enzyme.

The explanations given for the inability of the T.foetus enzymes to completely remove monosaccharide residues from the acetylcholine receptor are also relevant to the results of mixed endoglycosidase D enzyme treatment. This enzyme treatment produced only a 40% reduction in the total amount of sugars present in the receptor. The mixed endoglycosidase D enzymes consisted

of neuraminidase, β-galactosidase, β-N-acetylglucosaminidase and endoglycosidase D. This mixture of enzymes has been used by Muramatsu et al (1978) to release neutral sugars from glycoproteins (69%, 68% and 83% of the neutral sugars were removed from transferrin, fetuin and γ-globulin respectively). Endo-β-N-acetylglucosaminidase D (from D.pneumoniae) is an enzyme which cleaves oligosaccharides of the complex type, such as those found in IgG, transferrin and fetuin, provided that peripheral sugars have been removed by treatment with exoglycosidases. The general structure of susceptible substrates is shown below.



R_1 = H, monosaccharides or oligosaccharides.

R_2 = peptides or proteins.

* indicates the point of cleavage.

(from Muramatusu et al., 1978)

The fact that endoglycosides D treatment of AChR releases mannose suggests that at least part of the oligosaccharide (from one or more of the subunits) of Torpedo acetylcholine receptor has the core structure above which is exposed by the action of galactosidase, glycosidase and neuraminidase. The results of enzyme

treatment indicate that there is either structural heterogeneity of the total sugar content of the receptor or there is a heterogeneity of the monosaccharide content between the subunits. There may, in fact be a combination of the two types of heterogeneity.

Characterisation of the oligosaccharide chain of the α-subunit of the acetylcholine receptor of the clonal mouse muscle cell line BC3H-1 in terms of their sensitivity to endo-β-N-acetylglucosaminidase H and by comparison of the native glycosylated polypeptide with the non-glycosylated form made in tunicamycin-treated cells has been carried out (Merlie et al., 1981). These studies indicated that the native α-subunit has a single N-asparagine-linked oligasaccharide chain of the high-mannose type. This evidence points to the second suggestion (heterogeneity between subunits) being correct.

5.3.4 Effect of Alkaline Borohydride Treatment on the Sugar Content of Torpedo AChR

Prolonged alkaline borohydride treatment produced no apparent release of sugars from the acetylcholine receptor. As oligosaccharides O-glycosidically linked to serine or threonine are alkali labile it must be assumed that the resistance of the monosaccharides to cleavage under alkaline conditions is due to their being linked N-glycosidically. Possible sites for N-glycosidic linkage have been proposed (Noda et al., 1982, 1983a,b)

(see Introduction).

5.4 Development of a Lectin-Binding Assay for Glycoproteins Immobilised on Nitrocellulose Paper

Lectins potentially provide a very powerful tool for elucidating the oligosaccharide structure of a glycoprotein as they bind specifically to certain saccharide structures. Some lectin binding studies have previously been carried out on the acetylcholine receptor. These studies have indicated that the AChR interacts with lectins from Canavalia ensiformis (Con A) (mannose), T.vulgaris (N-acetyl-D-glucosamine), Lens culinaris (mannose) (Meunier et al., 1974), R.communis (galactose) (Michaelson et al., 1974) and Helix pomatia (N-acetylgalactosamine) (Bersinger et al., 1983a). mAChR (porcine atrial muscle) has been shown to bind to T.vulgaris, R.communis, Con A and G.max (N-acetylglucosamine, Herron and Schimerlik, 1983). These investigations of the carbohydrate nature of the acetylcholine receptor have been carried out by using lectins immobilised on columns, radiolabelled lectins or have relied on the ability of Con A to bind mannose present in horseradish peroxidase.

Because of the need to examine the subunit distribution of monosaccharides in only small amounts of AChR glycoprotein, a lectin-binding assay was developed to probe nitrocellulose blots of SDS polyacrylamide gels.

A previously used method of examining the subunit

sugar distribution of the AChR has involved SDS PAGE and subsequent staining of the gel by using radiolabelled lectins (Wennogle and Changeux, 1980). Examination of the carbohydrate nature of other glycoproteins has also been achieved by staining gels with FITC-labelled lectins (Furlan et al., 1979). Both these techniques depend on the lectins' diffusing through the gel and their eventual interaction with sugar residues. There are several problems with both types of labelled lectins. For example, diffusion of the lectin if possible, takes a long time, as does removal, by washing, of unbound lectin. There are moreover, problems encountered in visualising the bound lectin. FITC-labelled gels depend upon visualisation by using a U.V. source and are consequently difficult to photograph. Radiolabelled gels must be either sliced and the radioactivity counted or subjected to autoradiography. Radiolabelled lectins are also hazardous to handle. The technique presented here is simpler to perform, non-specific binding can be removed more easily and there is immediate visualisation of positive reactions.

The method developed involves blotting the proteins or glycoproteins onto nitrocellulose sheets by using the modified method of Bittner et al (1980). Visualisation of the bound lectin is achieved by incubation with a glycoprotein conjugated to horseradish peroxidase. As lectins have at least two binding sites they are able to bind the immoblised glycoprotein on the one hand and the enzyme-labelled glycoprotein on the other. The advantage

in labelling a glycoprotein and not the lectin directly is that the labelled glycoprotein can be used for the detection of a number of lectins.

Following conjugation of horseradish peroxidase to various glycoproteins these conjugates were tested for their ability to bind lectins. The results of this test showed the binding of lectins to the conjugates to be specific.

The Fcγ-HPO conjugate reacted with all the lectins tested. The next best conjugate was the thyroglobulin lectin which failed to show a reaction with Ulex and Phaseolus lectin. There are two possible reasons for the advantages of the Fcγ conjugate. The first is that Fcγ has a higher percentage of sugar (18%) than thyroglobulin (9%), and the second is that Fcγ has 2 carbohydrate side chains, whereas the carbohydrate of thyroglobulin is a single terminal chain.

Specificity of the reaction was assured as no reaction was produced by incubation of the lectins with the substrate only and, incubation with horseradish peroxidase alone produced a positive reaction with only Con A and, to a lesser extent, with Pisum lectin (also specific for mannose).

A similar technique has been developed by Kieda et al (1977). In this method glycopeptides from thyroglobulin were labelled with HPO and ferritin. The porcine thyroglobulin-HPO marker was found to precipitate (in a double-diffusion assay) Con A, Phaseolus, G.max, Arachis* and Ricinus* (* following desialation of the

thyroglobulin glycopeptide). They reported no precipitation of either T.vulgaris or Limulus lectin with the conjugate. The ability of the conjugate to precipitate fucose-binding lectins was not tested. Kieda et al (1977) stated that the thyroglobulin glycopeptide requires desialation for it to react with Ricinus and Arachis as these lectins only react with unsubstituted galactose residues. Due to the presence of N-acetylglucosamine and sialic acid in the thyroglobulin in is suprising that Kieda et al (1977) failed to detect any reaction with T.vulgaris lectin.

Lectin binding to saccharide structures is, in many instances, very complex and difficult to interpret. Whilst lectins are often quoted as binding particular monosaccharides their specific targets are often di-, tri- or oligosaccharides and binding may require the presence of monosaccharides some distance from the specific binding group. This often means that the interpretation of results of lectin binding studies has to be treated very carefully. In some instances, the fact that an oligosaccharide structure fails to bind a particular lectin does not necessarilly mean that it does not contain a particular sugar, it is possible that the monosaccharide is incorrectly attached for specific lectin binding to occur.

5.4.1 Detection of Lectin Binding to AChR Using Glycoprotein-HPO Conjugates (Spot Test)

The spot test is a simple method of detecting lectin binding saccharide structures in a glycoprotein. In this test increasing amounts of *Torpedo* acetylcholine receptor was spotted onto nitrocellulose and incubated with various lectins. The control (BSA) produced no reaction on incubation with lectins demonstrating that the test was specific for glycoproteins. The lectin binding was identified by incubation with the thyroglobulin-HPO conjugate. Results of this test confirmed the results of gas-liquid chromatography analysis and sialic acid assays regarding the carbohydrate nature of the receptor. Binding of *Lotus*, Con A, (*Pisum*, *Lens*), *Arachis*, *Triticum* and *Limulus* indicate the presence of fucose, mannose, galactose, N-acetylglucosamine and sialic acid. Also, specific binding of *Lens* and *Pisum* lectins requires the presence of fucose in the saccharide structure (Kornfeld *et al.*, 1981).

The receptor was also found to bind to *Glycine max*, reputedly specific for N-acetylgalactosamine, however, this monosaccharide was not demonstrated by GLC analysis. The lectin from *Glycine max* is unique among the lectins tested as the best inhibitor of haemagglutination induced by this lectin is the monosaccharide N-acetyl-galactosamine (GalNAc). Other saccharide structures which have been shown to inhibit binding of this lectin

include lactose and galactose (though with reduced effect compared with GalNAc). At this stage it is difficult to try to interpret these results in terms of any possible saccharide structures that may be present in the receptor.

5.5 Subunit Distribution of AChR Lectin Binding Components

Periodate-Schiff staining of the subunits of the acetylcholine receptor following SDS gel electrophoresis produced four positively-stained bands, confirming the results of others (Raftery et al., 1973, Karlin et al., 1975, Vandlen et al., 1979). While this staining indicates that all the subunits are glycosylated it gives no indication as to the amount or type of monosaccharides present in each chain. By means of the glycoprotein-HPO conjugate test the subunit distribution of sugar residues can be determined through their interaction with lectins.

Various proteins were run on gradient polyacrylamide gels and blotted onto nitrocellulose paper (proteins of widely differing molecular weight can be transfered equally well). Nitrocellulose sheets from these gels were treated as with the spot test. Of the standard proteins run on the gels all were visible when stained for protein (amido black) as were the four subunits of the AChR showing that the proteins were transfered efficiectly.

1) Mannose/Glucose subunit distribution

Through the binding of Con A, mannose was shown to be present in all four subunits, a result which has been previously reported (Criado and Barrantes, 1982). Unlike Con A, the Pisum sativum lectin (Pea) bound to only the

γ- and δ-chains. At first sight this is somewhat surprising as this lectin also has α-glucosyl- and α-mannosyl-binding specificity (Van Wauwe et al., 1975, Allen et al., 1976). However, it has been shown that the presence of a fucose residue in the carbohydrate structure is essential for high affinity binding of this lectin (Kornfeld et al., 1981) and its failure to bind all subunits may not so much indicate the absence of mannose as the absence of fucose from the α- and β-chains (see below).

2) Subunit distribution of galactose

Through Arachis lectin binding the presence of galactose was detected in the γ- and δ-chains only. Subunit distribution of galactose has only been reported once before (Lindstrom et al., 1979) when it was shown that all subunits contained galactose. While their results conflict with those reported here, Lindstrom et al (1979) did show that the γ- and δ-subunits contained 4x and 2x the galactose content of the α- and β-subunits. Lindstrom's observations were obtained from a single set of gas-liquid chromatographic analysis.

3) Subunit distribution of Fucose

As with galactose, fucose was detected in only the γ- and δ-subunits (through binding with the Lotus tetragonolobus lectin). While the presence of fucose in the AChR has not been published, its presence has been shown previously in this laboratory and the Lotus lectin

binding observed here confirms the results of GLC analysis discussed earlier. The detection of fucose in only two of the subunits appears to be supported by the results obtained following incubation with the Pea lectin (see earlier section).

4) Subunit distribution of N-acetylglucosamine and sialic acid

While Phaseolus vulgaris lectin binding to AChR suggests that only the γ- and δ-subunits contain N-acetylglucosamine (Wennogle and Changeux, 1980), Lindstrom et al (1979) have demonstrated by GLC analysis that all four subunits contain this sugar. Incubation of AChR blots with Triticum lectin suggested the presence of GlcNAc in the γ- and δ-subunits only.

Triticum lectin has been shown not to bind to chitobiose residues when present in oligosaccharide chains despite the fact that this residue is a potent inhibitor of lectin induced haemagglutination (Debray et al., 1981). Thus, the failure of this lectin to bind to the α- and β-subunits may not necessarily reflect the absence of GlcNAc.

Triticum has been shown to bind to terminal N-acetylneuraminic acid, and it is therefore conceivable that the above results may arise from presence of these residues. While the succinylated Triticum lectin does not bind sialic acid (Monsigny et al., 1979) its binding to GlcNAc and GlcNAc-β-(1$\rightarrow$4)-GlcNAc is very similar to that of the native lectin. Incubation of the

succinylated lectin with AChR showed binding to only the γ- and δ-chains, confirming that at least some of the reaction of Triticum lectin with the receptor subunits was via GlcNAc residues.

Similar results have been obtained for the subunit distribution of sialic acid. One investigation has shown only the γ- and δ-chains to have sialic acid (Lindstrom et al., 1979) while another has shown its presence in all subunits (Vandlen et al., 1979). As stated previously the Triticum lectin does show some binding to sialic acid and incubation of this lectin with the receptor showed binding to the γ- and δ- chains only. Neuraminidase treatment of the AChR resulted in a reduction in the intensity of binding of the lectin over that of the untreated receptor, suggesting that part of the binding was through sialic acid residues. Further evidence to support the presence of sialic acid in just two of the subunits has come from Criado and Barrantes (1982) who showed that neuraminidase treatment of Torpedo AChR enhanced the electrophoretic mobility of the γ- and δ-chains only.

It appears, therefore, that the acetylcholine receptor from Torpedo exhibits heterogeneity of its carbohydrate content and that the α- and β-subunits contain mannose (and possibly N-acetylglucosamine) only and the γ- and δ-chains contain these monosaccharide residues plus fucose, galactose and sialic acid. The presence or absence of glucose in the subunits is

difficult to confirm.

5.6 Enzyme-Linked Lectin Binding Assay (ELBA)

This type of lectin binding assay has not been explored before using the acetylcholine receptor. Others have shown that a known lectin receptor (eg ovalbumin) can be adsorbed to a solid phase without losing its lectin binding properties (van der Schaal et al., 1984). From these results it was concluded that both small mono- or disaccharides and larger polysaccharides or glycoproteins, with one or more lectin binding site, could be tested. Also, the senstitivity of the ELBA was equal to or higher than other assays previously investigated. This assay was adapted to examine the ability of the AChR to bind lectins, the test substance (AChR or another glycoprotein) being adsorbed onto cuvettes followed by incubation with a lectin solution.

1) Detection of mannose/glucose residues using the ELBA

As a control, Con A was tested for its ability to interact with the test substances (IgM, gelatin and native and neuraminadase treated AChR) as it will also bind horseradish peroxidase (the enzyme marker used for visualisation of lectin binding). The failure of Con A to bind gelatin gives an indication of the specificity of the assay. Although IgM has a low percentage of sugars (approximately 10%) it was found to bind increasing

amounts of Con A and to saturate around 12μg/ml lectin. Con A also bound Torpedo AChR with equal affinity. Neuraminidase treatment of AChR had no effect on its lectin binding capability. The binding of Con A to IgM and AChR shows the presence of mannose. Binding of Con A to glycoproteins could be specifically inhibited (100%) by coincubation with mannose/glucose.

2) Detection of fucose residues using the ELBA

Lotus tetragonolobus lectin (specific for some fucose residues) will not bind horseradish peroxidase (HPO), therefore, commercially available Lotus lectin coupled to HPO was used to detect fucose residues in glycoproteins. Assays performed, as for Con A binding, were shown to be specific by the failure of the lectin to bind gelatin. IgM was used as a positive control for the assay. Lectin binding to IgM was saturable around 14μg/ml.

The binding of Torpedo acetylcholine receptor to the Lotus lectin confirmed the results of gas-liquid chromatography and previous lectin binding studies. The binding was saturable at around 10 μg/ml, predictably lower than that of IgM which has a higher percentage of sugar than the receptor.

Neuraminidase treatment of the acetylcholine receptor showed that no fucose had been masked by sialic acid.

Lotus-HPO lectin binding to acetylcholine receptor

coated cuvettes could be inhibited by increasing amounts of L-fucose but not D-glucose. This binding could also be inhibited by free receptor and IgM. Fetuin produced no inhibition of the binding. Free acetylcholine receptor failed to produce 100% inhibition of binding, the inhibition appeared to plateau at around 65 - 70%, this may be due to steric hindrance as the AChR is a very large molecule (M.W. 250,000).

5.7 Inhibition of lectin-induced haemagglutination with Torpedo acetylcholine receptor

Debray et al (1981) demonstrated inhibition of lectin-induced haemagglutination by using various saccharide structures (including mono-, di-, tri- and oligosaccharides and glycopeptides). In general, monosaccharides produced complete inhibition of haemagglutination when present in mM concentrations whereas many oligosaccharides and glucopeptides required only μM amounts.

The lectins used in this particular inhibition experiment were chosen as they readily agglutinated type O red blood cells. Inhibition of erythrocyte agglutination has proved to be a simple and rapid means of indicating the presence of saccharide structures in a glycoprotein especially when the glycoprotein in question is available in very small amounts. The assay has proved to be much more sensitive than the enzyme-linked lectin binding assay (ELBA) discussed earlier, requiring only

pMolar amounts of receptor. In comparison with the results of Debray et al (1981), the results presented here appear to be in the correct order of magnitude for a glycoprotein to inhibit lectin haemagglutination.

With the exception of that caused by Ulex lectin, Torpedo acetylcholine receptor caused complete inhibition of lectin-induced haemagglutination when present in pM amounts. The failure of Ulex-induced haemagglutination to be inhibited by the AChR may be a reflection of the binding specificity of the lectin and thus the oligosaccharide structure of the receptor. The addition of a second L-fucose α-(3)-linked to an N-acetylglucosamine or glucose residue reduces the inhibitory power of an oligosaccharide (Debray et al., 1981). Con A-induced haemagglutination could be inhibited with as little as 1pM AChR. The acetylcholine receptor also inhibited G.max-induced haemagglutination. This interaction of the receptor with this lectin has been shown before and explained in terms of its affinity for galactose residues as well as N-acetylglucosamine.

5.8 Isoelectric Focussing of AChR and Effect of Enzyme Treatment

Isoelectric focussing of the AChR in neutral detergent gives a broad peak centered around pH 5.2 (α-toxin complex, Biesecker, 1973, Raftery et al., 1971) or 4.8 (free receptor, Biesecker, 1972, Heilbronn et al., 1974). Determination of the pI value of the

receptor-α-toxin complex gave a value of 5.22 ± 0.10, which is identical to that reported in the literature. Neuraminidase treatment of the receptor produced a pI value of 5.4, this was a notable increase over that of the untreated receptor. As sialic acid is very electronegative its removal would be expected to alter the Pi value of the AChR. To my knowledge, there has been no investigation of the effect of enzyme treatment on the isoelectric focussing point of the receptor, although neuraminidase treatment has been shown to alter the electrophoretic mobility of two of the subunits on polyacrylamide gels (Criado and Barrantes, 1982).

5.9 Determination of the Molecular Weight of Torpedo AChR

Determination of the molecular weight of the subunits of Torpedo acetylcholine receptor (SDS polyacrylamide gels, 7.5% (w/v)) produced values of 42K, 49K, 57K and 62K (for the α-, β-, γ- and δ-subunits respectively). The values are in good agreement with those of Raftery et al., 1980 (40K, 50K, 60K and 65K for the α-, β-, γ- and δ-subunits respectively).

5.10 Passive Haemagglutination Test

The main problem encountered when solubilised membrane proteins are coupled to erythrocytes is the presence of detergents which, presumably, would cause haemolysis. Prior fixation of the cells may overcome detergent-induced haemolysis, and recently, Steinitz and Tamir (1985) coated glutaraldehyde-fixed red cells with proteins in the presence of the ionic detergent deoxycholate. However, other workers found that fixed cells could not be coated by the chromic chloride method (Ling et al., 1979). The results presented here show that, at least in the case of non-ionic detergent-solubilised membrane proteins, dialysis was sufficient to reduce the level of detergent to below that causing cell lysis. Dialysis was preferred to other methods of detergent removal because it does not effect the protein-bound detergent and therefore the protein integrity is preserved.

The chromic chloride procedure was successfully used to couple solubilised Torpedo AChR to red blood cells. The binding of AChR to the red blood cells was confirmed by the binding of $^{125}I\alpha$BGT. Binding of αBGT to the bound AChR gave an apparent Kd of 6.2nM and a B_{max} of 0.8pmoles which are comparable to values given for the receptor in electoplax membranes (9.0 $\pm$ 2.2nM (Lukasiewicz et al., 1978). The similarity in values for

Kd obtained for the bound and free receptor indicate that at least the toxin binding site had not been damaged by the coupling procedure. This apparent lack of damage to the toxin-binding site, following coupling of solubilised AChR, means that this test could offer a convenient and simple assay for studying ligand-receptor interactions. In such studies, the separation of bound from free cholinergic ligands is usually carried out by using anion-exchange DE52 cellulose filters, ammonium sulphate precipitation or gel filtration (Schmidt and Raftery, 1973).

The coated cells behaved well in the agglutination test and were used to detect anti-AChR antibodies with a high level of sensitivity. Correlation between the agglutination test and radioimmunoassay was quite good in the case of polyclonal antisera. The anomalous results obtained with monoclonal antibodies are not unexpected. In contrast to polyclonal antisera, monoclonal antibody-antigen binding is greatly dependent on the type of assay used. The generally low agglutination titre obtained is most likely due to the fact that the monoclonal antibodies are directed to single antigenic determinants and therefore their cross-linking capability is very much less than that of a polyclonal antiserum. The relatively high agglutination titre obtained with mAb-C7 when compared with mAb-A3 (see Table 4.9.2) is a reflection of difference in affinity (another important factor in monoclonal antibody-antigen reaction). Kinetic

studies (not presented here) have shown that C7 had a higher binding affinity to Torpedo AChR than A3.

SUGGESTIONS FOR FURTHER WORK

Gas liquid chromatographic analysis has provided a good estimation of the total sugar content and relative amounts of monosaccharides present in the intact Torpedo AChR. However, to confirm the results of lectin binding studies on the subunit distribution of sugar residues GLC, analysis of the chains should be carried out. Separation of the subunits by SDS-PAGE and their subsequent removal from the gel followed by GLC would provide quantitative data on the monosaccharide content of each chain.

With large quantities of intact AChR or individual subunits (>10mg), sequential incubation with a number of glycosidases coupled with GLC analysis would provide information on the oligosaccharide structure. Such information could also be obtained by premethylation of free hydroxyl groups, followed by acid hydrolysis and gas-liquid chromatographic analysis of the partially methylated monosaccharides.

The development of the assay for the detection of lectin binding components in protein blots using HPO-glycoprotein conjugates enabled the subunit distribution of sugar residues in the AChR to be elucidated. This assay may also prove useful in establishing whether or not some sugar residues are antigenic by looking for inhibition of lectin binding by antibodies to AChR.

The enzyme-linked lectin binding assay (ELBA) has proved useful here in confirming the presence of fucose in the Torpedo AChR. This assay should, however, be examined further using other lectins and in combination with glycosidase treatment of the receptor.

The passive haemagglutination test has proved a very rapid method for the detection of anti-(nAChR) antibodies. This technique can be useful in the isolation of specific B-lymphocytes, in ligand interactions with AChR and in the characterisation of monoclonal antibodies to AChR in addition to RIA and ELISA. This assay also has a potential for the investigation of lectin binding to AChR. The use of cells of specific blood groups and/or treatment of the cells with periodate (Blumenfeld et al., 1972) prior to coating with AChR would prevent non-specific lectin binding. As with the analytical techniques mentioned above, this assay could also be use for the detection of possible anti-(AChR carbohydrate) antibodies in polyclonal sera from various sources.

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6. ADDENDUM TO INTRODUCTION - Recent information on the structure, function and subunits of the acetylcholine receptor

6.1 Subunit Structure and Function

The complete primary structure predicted from the sequences of four separate cDNAs has confirmed the existence of four distinct but highly homologous subunits (α β, $\gamma(\varepsilon)$, δ) containing approximately 500 amino acids each (Numa et al., 1983). The considerable homology relationships among the four is somewhat surprising and suggests that all were derived from a common ancestral gene.

Whilst it has been confirmed that acetylcholine binds to the α-subunit, precise location of the binding site has been difficult to determine. Noda et al (1982) initially proposed that cysteines α128 and α142 were linked by a disulphide bond and that the resulting loop formed the acetylcholine binding site. Work by Oblas et al (1986) appears to confirm this. However, using monoclonal antibodies directed against sequence α127-143 of the α-subunits Criado et al (1986) have shown that this is not the case. Criado's group were unable to inhibit the binding of anti-synthetic peptide α127-143 monoclonal antibodies to immobilised receptors by using cholinergic ligands. In addition these monoclonal antibodies when bound to the receptor did not affect

toxin binding kinetics.

Investigation of the α-subunits following V8 protease digestion indicates that they may differ in their degree of glycosylation and in their ability to bind cholinergic ligands (Lindstrom et al, 1983, Conti-Tronconi et al, 1984, Pedersen et al, 1986, Ratnam et al, 1986a, Oblas et al, 1986).

The most recent and somewhat surprising advancement in knowledge concerning the acetylcholine receptor has been the discovery of another subunit. A fifth subunit cDNA (ε) has been discovered in calf muscle (Takai et al, 1985). The ε-subunit shares structural features common to all four subunits especially with respect to transmembrane orientation and possible glycosylation sites. In addition to position 141 (common to all subunits) the calf ε-subunit contains two additional potential N-glycosylation sites (aligned at positions 66 and 307) one of which (position 66) is assigned to the extracellular side of the membrane. The ε-subunit will substitute for Torpedo γ-subunit in the oocyte expression system. N.B. All four AChR subunits must be present for a functional channel to be assembled.

The discovery of the ε-subunit appears to go a long way to explain the differences between embryonic and adult type AChRs. Early biochemical studies suggested that embryonic and adult type AChRs differ in their structure, and indeed certain antibodies can distinguish between two types of channels (Schuetze et al, 1985).

Differences were thought to be due to either different gene products or due to the embryonic-type AChR being converted into adult-type ones via a post-translational modification. From the oocyte expression system Mishina et al (1986) have shown that high levels of γ-subunit mRNA are found only prenatally and conversely, high levels of ε-subunit message are found only postnatally. Late in gestation, muscles express significant levels of both messages. This suggests that the embryonic AChR has an $\alpha_2\beta\gamma\delta$ subunit composition, whereas the adult AChR has an $\alpha_2\beta\varepsilon\delta$ subunit structure.

The substitution of the γ-subunit for the ε-subunit appears to explain differences in the operational characteristics of embryonic and adult AChR. When oocytes were injected with a mixture of α, β, γ, and δ-subunit mRNAs, they expressed channels that had the same prolonged open time and low conductance characteristics of AChRs in embryonic bovine muscle (Mishina et al, 1986). Conversely, oocytes injected with α, β, ε, and δ-subunit mRNAs expressed AChRs with the brief open time and high conductance typical of channels in adult end plates.

Developmental change in gene expression directly effects changes in AChR channel properties in bovine muscle. This is most likely as a consequence of innervation as denervation of rat muscle at birth inhibits the appearance of adult type channels (Schuetze and Vicini, 1984).

Recent work by Sakmann et al (1985) suggests that the δ-subunit determines the channel-closing step of the operation of the AChR. They found that substitution of any of the subunits of Torpedo AChR with the corresponding subunit from calf receptor in oocyte membranes could be achieved to form functional channels. In contrast, however, the gating behaviour differed widely in calf and hybrid AChR channels. The amount of bovine δ-subunit mRNA has (like the γ-subunit) been shown to fall to a low but detectable level at postnatal stages in development. There is a possibility that this subunit, like the γ-subunit, is also replaced.

6.2 Function of Phosphorylation of the AChR

Phosphorylation of the receptor preparation from T.californica appears to increase the rate (by many fold) of one phase of desensitisation (Steinback and Zempel, 1987). Desensitiation of the AChR is a phenomenon in which the receptor becomes unresponsive to activation when it is exposed to an activating ligand for a relatively long period of time. Desensitisation results in AChR whose channels remain closed even when acetylcholine is bound to the receptor.

6.3 Structure of the AChR within the membrane

As already stated in the introduction, a theoretical model of the transmembrane orientation of the

polypeptide chains of the receptor indicates that there are four hydrophobic domains (M1 - M4) and one amphipathic (MA) transmembrane domain. The MA domain was predicted to form an α-helix with polar residues on the one side and hydrophobic residues on the other, so that corresponding domains from the five subunits could come together like barrel staves and form a channel with a hydrophilic lining (Guy, 1983; Finer-Moore and Stroud, 1984). However, work by Hucho et al (1986) suggests that the ion channel of the AChR is lined by helix II (M2). Comparing the known sequences of AChR subunits from a variety of organisms, helix II appears to be better conserved than MA. Hucho et al (1986) point out that, although the M2 domain may at first site appear too hydrophobic to form a water-filled pore (Lewis and Stevens, 1983) this is only in the upper part.

Evidence has been found for two additional amphipathic transmembrane domains (M6 and M7) between the N-terminus and M1 (Criado et al, 1985). Further information can be obtained from reviews by Ratnam et al (1986) and Merlie and Smith (1986).

6.4 Carbohydrate Content of the AChR

Recent investigations (Nomoto et al, 1986) have revealed considerable information on the carbohydrate structure of the AChR from Torpedo californica. Treatment of the receptor with N-oligosaccharide

glycopeptidase showed the sugars to be N-linked to the protein. Nomoto's group showed that 70% of the total oligosaccharide chains to be of the high-mannose type with the structure $(Man)_8(GlcNAc)_2$. N-Acetyl-galactosamine was not detected thereby suggesting that no sugar residues were linked to the protein O-glycosidically. The carbohydrate composition in one molecule of Torpedo AChR was found to be 28, 53, 2.0, 11 and 4.9 residues for GlcNAc, Man, Fuc, Gal and NeuAc respectively. The percentage of high-mannose-type oligosaccharides was greatest in the α- and β-subunits. It was found that, in the γ- and δ-subunits less than half of the total oligosaccharides were of the high-mannose-type, the rest being a variety of the complex-type. Nomoto *et al* estimate numbers of oligosaccharide units in each subunit as one, one, two and three for α, β, γ and δ subunits respectively.

7. ADDENDUM TO DISCUSSION

Conclusions drawn by the work presented here are that the carbohydrate structure of the Torpedo AChR consists of both high-mannose-type and complex-type oligosaccharide chains. Although it is difficult to conclusively determine their subunit distribution, experiments reported here and by Nomoto et al (1986) suggest that high-mannose chains are present on all four subunits whereas complex-type chains are present on the γ- and δ-subunits only. The suggested subunit distribution of carbohydrates in the AChR is illustrated in Figure 7.1. While one of the α-subunits has been omitted from the diagram it is likely that it is glycosylated in a similar manner. However, various investigators (Conti-Tronconi et al., 1984; Lindstrom et al., 1983; Oblas et al., 1986) have indicated that the two α-subunits may differ in their degree of glycosylation.

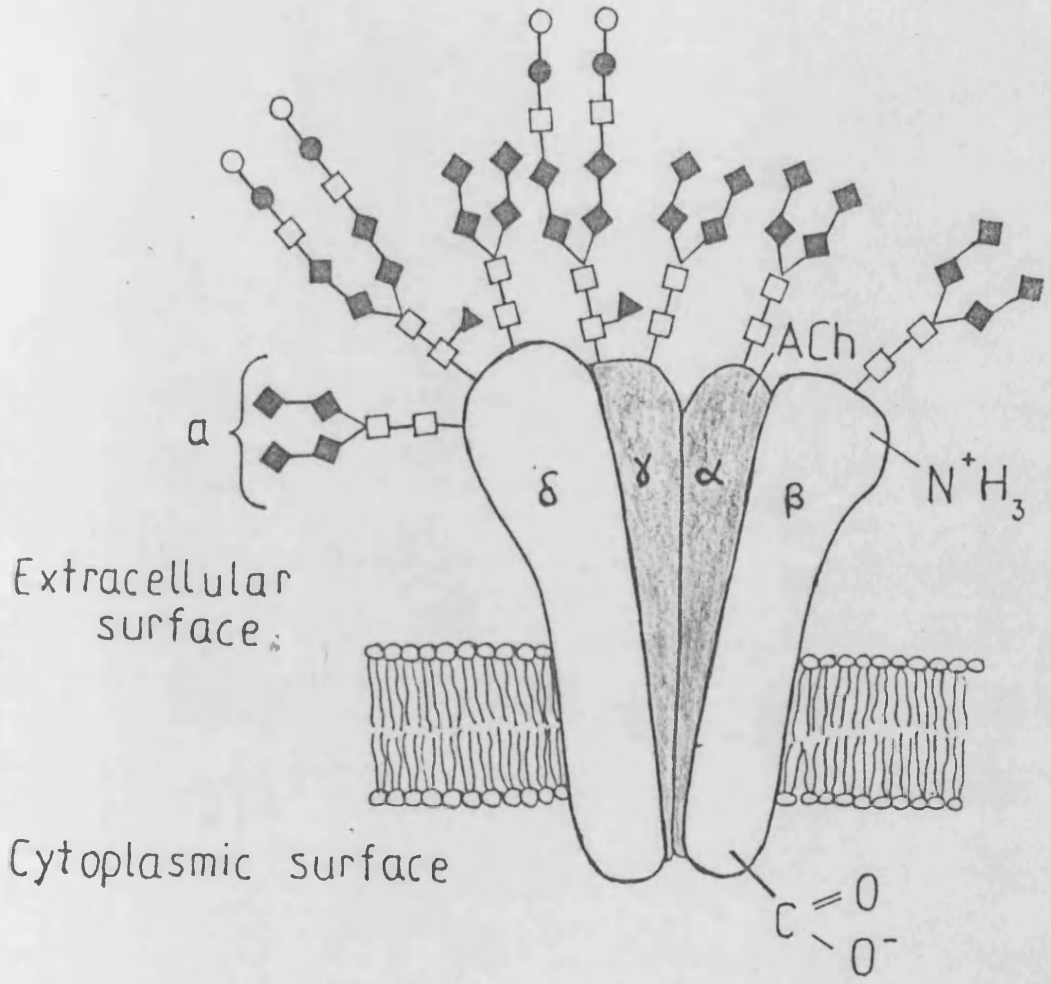
Glucose residues were occasionally found in AChR prepared for this work and by other workers (Nomoto et al., 1986). The presence of glucose due to contamination has been ruled out (see page 201). During glycosylation of N-linked glycoproteins, an oligosaccharide structure consisting of $(GlcNAc)_2$-$(Man)_9$-$(Glc)_3$ is transfered to the protein backbone (Hubbard and Ivatt, 1981). This structure is subsequently posttranslationally modified

Figure 7.1 Diagramatic representation of the subunit distribution of oligosaccharides in the Torpedo AChR

One of the α-subunits has been removed for clarity but maybe essentially glycoyslated in the same manner as the α-subunit shown. All sugar residues appear to be present on the extracellular surface of the membrane and are attached to an asparagine residue.

a - This oligosaccharide may be of a high-mannose or complex-type structure.

ACh - Acetylcholine-binding site.



High-mannose type oligosaccharide

Complex type oligosaccharide

□ – GlcNAC ◆ – Man ● – Gal ○ – NeuNAc ▶ – Fuc

one step of which involves the removal of the glucose residues. The glucose in receptor preparations may therefore be present following the purification of functionally mature AChR which had not undergone complete modification of its carbohydrate component.

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A Passive Haemagglutination Test for the Detection of Anti-Nicotinic Acetylcholine Receptor Antibodies

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A detergent-solubilised membrane protein (the nicotinic acetylcholine receptor) was coupled to red blood cells by the chromic chloride method. The bound receptor retained both antigenic activity and the ability to bind alpha neurotoxins. Coated cells were successfully used in a haemagglutination test to determine antibody titre and cross-reactivity of polyclonal and monoclonal anti-acetylcholine receptor antisera.

Key words: *acetylcholine receptor – antibodies – passive hemagglutination*

Introduction

Red blood cells coated with antigens or antibodies by the chromic chloride method (Goding, 1976) have proved to be of great value in agglutination, complement-mediated lysis, and rosette assays (reviewed by Coombs, 1981). Antibody-coated erythrocytes have been used for the detection of surface immunoglobulins (Dhaliwal et al., 1978), major histocompatibility antigens (Binns et al., 1983) and monoclonal antibodies to cell surface antigens (Clark et al., 1984). Antigen-coated red cells have been used in haemagglutination tests to determine specific antibody titres and have a sensitivity comparable to that of radioimmunoassay (Coombs, 1981). They have also been used in the rosette reaction to isolate specific antibody-secreting lymphocytes (Steinitz and Tamir, 1980).

Coating of red cells with cell surface proteins has not been reported (to our knowledge) since ionic and non-ionic detergents which are normally used in the extraction of membrane proteins would be expected to lyse red cells. However, we have observed that sheep and ox red blood cells were not lysed by the widely used non-ionic detergent Triton X-100 at concentrations below 0.1% (v/v). We report here the coupling of a membrane protein, the nicotinic acetylcholine receptor (AChR) from *Torpedo marmorata,* to sheep red blood cells. The bound receptor retained both antigenic activity and the ability to bind the nicotinic antagonist α-bungarotoxin (αBGT). The coated cells were successfully used to determine the antibody titre, cross-reactivity and complement-mediated lytic activity of polyclonal

and monoclonal anti-AChR antisera with sensitivity comparable to that of the routinely used double antibody radioimmunoassay (Lindstrom, 1977).

Antibodies to the nicotinic AChR have been demonstrated in the autoimmune disorder myasthenia gravis (MG) where they are present in 80–90% of the patients (Lindstrom et al., 1976). These autoantibodies are usually measured by a radioimmunoassay which depends upon the precipitation of ^{125}I-αBGT-labelled AChR-antibody complexes either with a second antibody (Lindstrom, 1977) or by *Staphylococcus aureus* cells (Tindall et al., 1981). An enzyme-immunoassay may also be used with horse-radish peroxidase-labelled αBGT (Furukawa et al., 1984). Alternative enzyme-linked immunosorbent assays (ELISA) have recently been developed (Norcross et al., 1980; Dwyer et al., 1983; Hinman et al., 1983; Kawanami et al., 1984). The procedure described here may offer a further alternative for assaying anti-AChR antibodies. The method is rapid, very reproducible and does not require any special equipment (gamma counter, ELISA readers, etc). AChR-coated red cells may also be useful in the isolation of receptor specific B-lymphocytes by the rosette reaction.

Materials and Methods

Purification of acetylcholine receptor (AChR)

AChR was purified from the electric organs of *Torpedo marmorata* by detergent extraction and affinity chromatography as described by Lindstrom et al. (1981). Briefly, electric organ tissue was homogenised in 10 mM potassium phosphate buffer, pH 7.4, containing 5 mM EDTA and 20 mM sodium azide (homogenisation buffer), using a Sorvall Omnimix homogeniser. The homogenate was centrifuged at $20{,}000 \times g$ for 1 h at 4°C. The pellet was extracted in the homogenisation buffer containing 1% (v/v) Triton X-100 for 4 h at 4°C. The supernatant, containing the solubilised AChR, was collected after centrifugation at $100{,}000 \times g$ for 1 h at 4°C and applied to a column of *Naja naja siamensis* α-toxin (Miami Serpentarium, Florida) bound to Sepharose 4B. The bound receptor was eluted from the column with the homogenisation buffer containing 0.1% Triton X-100 and 10 mM benzoquinonium chloride (Stirling Winthrop, NY), and directly loaded onto a DEAE-cellulose column (DE-52 Whatman Biochemicals, Maidstone, Kent) equilibrated in the homogenisation buffer containing 0.1% Triton X-100. The receptor was eluted from the ion-exchange column using homogenisation buffer containing 0.1% Triton X-100 and 0.5 M NaCl and dialysed at 4°C against several changes of the same buffer without NaCl. The receptor solution was then concentrated using a Minicon concentrator (Amicon, Denver, CO) to give a protein concentration of 0.5 mg/ml as determined by a modified Lowry assay (Markwell et al., 1978) and sterilised by passage through a 0.22 μm Milipore filter. Aliquots were stored at 4°C or frozen at −20°C without loss of activity for at least 3 months. Purified AChR was obtained in mg quantities with 6–8 nmol of α-bungarotoxin (αBGT) binding sites/mg of protein as shown using the procedure of Schmidt and Raftery (1973). SDS-PAGE of the receptor revealed the presence of only the characteristic subunits with molecular weights of 40,000, 50,000, 60,000 and 65,000 (Raftery et al., 1980).

Attachment of acetylcholine receptor to sheep red blood cells (SRBC)

Fresh sheep red blood cells (SRBC, Tissue Culture Services, Slough, Berks) were coated with AChR by the chromic chloride procedure of Ling et al. (1977). The receptor was dialysed against saline (0.14 M NaCl) for 24 h before coating. 0.5 ml of a 10% (v/v) suspension of red cells was washed 5 times in saline by centrifugation ($400 \times g$, 5 min) and the supernatant discarded after the final wash. To the cell pellet was added 0.30 ml of AChR solution (0.15 mg) and, with vortex mixing, the required amount of 0.1 mg/ml aged chromic chloride ($CrCl_3 \cdot 6H_2O$, BDH Chemicals, Poole, Dorset) in saline solution (0.10–0.40 ml) was added. Vortexing was continued for a further 10 s, then saline (2 ml) was layered over the cells and the preparation left at 4°C overnight. The cells were then washed 3 times with Hepes-buffered Eagle's medium (containing 100 μg/ml streptomycin and 100 U/ml penicillin, H-Eagle, Flow laboratories, Irvine, Scotland) and finally suspended at 1% (v/v) in H-Eagle containing, 5% (v/v) heat-inactivated foetal calf serum (Flow Lab., H-Eagle-FCS), and stored at 4°C. Red cells coated with bovine haemoglobin (Hb, Sigma Chemical Co., Kingston-upon-Thames, Surrey) by the above procedure were used as controls.

Passive haemagglutination and complement-mediated lysis assays

Serial dilutions of antiserum were prepared in 0.05-ml volumes of H-Eagles-FCS in microtitre trays (Nunc, Gibco, Uxbridge, U.K.) 0.025 ml of 1% coated cells was added and the end-point read by the settled pattern technique after 2–4 h incubation at 20°C.

Complement-mediated lysis was set up as for the agglutination test with the following modifications: phosphate-buffered saline, pH 7.3, containing 1% bovine serum albumin (BSA/PBS) was used as the diluent and, before the addition of coated red cells, 0.025 ml of guinea pig complement (Miles, Slough, Berks, diluted 1:4 in BSA/PBS) was added. The microtitre plates were incubated in a humid chamber for 2 h at 37°C and the end-point (last well showing lysis) was recorded.

αBGT binding to AChR-coated red cells

α-Bungarotoxin (αBGT, Boehringer-Mannheim Corp., Lewes, Sussex) was radiolabelled with ^{125}I ($Na^{125}I$, specific activity 15 mCi/μg iodine, Radiochemical Centre, Amersham, Bucks.), by the chloramine T method of Hunter (1978) to a specific activity of 70 Ci/mmol.

Increasing amounts of ^{125}I-αBGT (0.034–4.77 pmol) were added to 0.020 ml aliquots of a 1% (v/v) suspension of coated red cells. The volume was made up to 0.2 ml with H-Eagle-FCS and the mixture incubated for 90 min at room temperature with occasional mixing. The cells were then washed 3 times ($400 \times g$, 10 min) with H-Eagle-FCS medium (0.5 ml) and bound radioactivity was counted in an LKB 1280 Ultragamma Counter. Specific binding (70–80% of total) was defined as binding of ^{125}I-αBGT that is displaceable by 63 mM benzoquinonium chloride.

Antisera

Polyclonal antisera were raised in rabbits and sheep by intramuscular injections of purified receptor (10–50 μg) in complete Freund's adjuvant followed by a similar

injection after 4 weeks. Blood was collected 5 days after the booster injection. Mouse monoclonal antibodies (mAb) to *Torpedo* AChR were prepared as described by Kearney et al. (1981). The monoclonal antibodies B11, C2 and C7 were used as culture supernatants. A3 was purified from ascitic fluid by affinity chromatography on a column of protein A bound to Sepharose CL4B (Pharmacia, Uppsala, Sweden). All antisera were heat-inactivated (30 min, 56°C) before use.

The titre of the anti-AChR antibodies was measured by the immunoprecipitation assay (Lindstrom, 1977), whereby the receptor is labelled with ^{125}I-αBGT then incubated with the antiserum and finally the toxin-receptor-antibody complex is precipitated with the appropriate second antibody. The titre is expressed in terms of receptor bound ^{125}I-αBGT precipitated per litre of antiserum.

Results

Effect of Triton X-100 on sheep red blood cells

Since AChR is solubilised by Triton X-100 extraction, the effect of this non-ionic detergent on SRBC was investigated. Serial dilutions of 1% (v/v) Triton X-100 were carried out in 0.05 ml volumes of saline in microtitre plates. 0.05 ml of a 1% (v/v) SRBC suspension in saline was added to each well and cell lysis observed after 2 h incubation at 20°C and 37°C. The detergent caused lysis at concentrations $\geqslant 0.1\%$ (w/v). The purified receptor preparations which contained 0.1% (v/v) Triton X-100 were dialysed for 24 h against saline to remove phosphate ions which inhibit $CrCl_3$ coupling (Goding, 1976) and also to remove excess detergent monomers. Dialysis does not remove protein-bound and protein-free detergent micelles (Furth, 1980) and, therefore, the receptor remains soluble and no aggregation of the protein is observed. Early attempts at removing the detergent by hydrophobic adsorption with Biobeads, Sm2 (Bio-Rad Labs., Richmond, CA) (Holloway, 1973) resulted in the aggregation and precipitation of the receptor and loss of αBGT binding activity. AChR dialysed against saline could be stored at −70°C without loss of activity for at least 3 months.

α-Bungarotoxin binding to AChR-coated SRBC

AChR is usually quantitated and characterised in terms of αBGT binding and, therefore, the ability of coated cells to bind this neurotoxin was determined. Fig. 1a shows that the bound receptor still retained toxin-binding activity. This was specific (displaceable by the cholinergic ligand benzoquinonium chloride). Haemoglobin-coated RBC showed negligible binding which was not displaceable by benzo-quinonium chloride. The Scatchard plot (Fig. 1b) gave an apparent K_d value of 6.2 nM and a B_{max} of 0.8 pmol which are comparable to values given for the receptor in free solution (Lukasiewicz et al., 1978).

Effect of the amount of chromic chloride used

The amount of $CrCl_3$ used influences both the uptake of protein and the agglutinability of the coated red cells. AChR uptake was measured in terms of

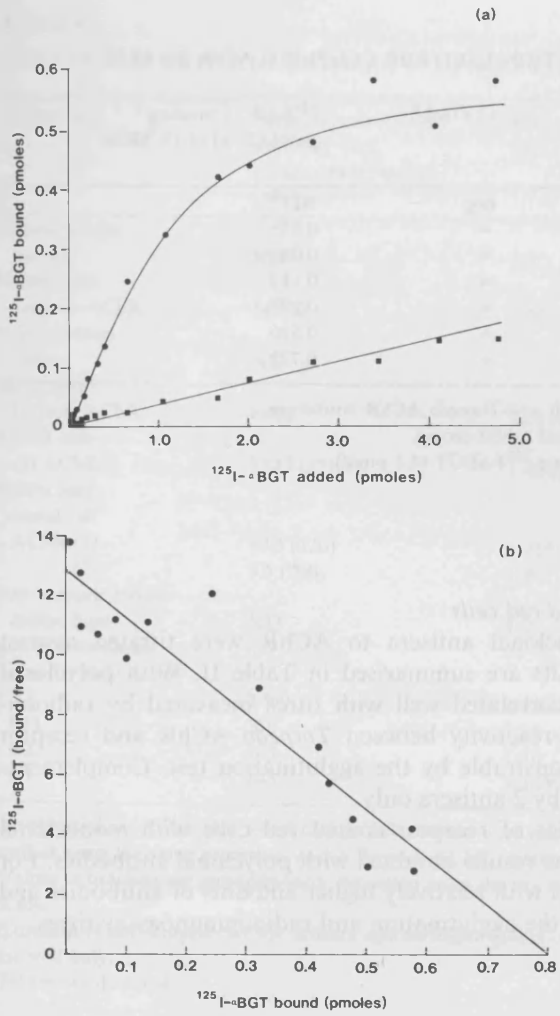

Fig. 1. ^{125}I-αBGT binding to AChR-coated red cells. a: increasing concentrations of ^{125}I-αBGT were incubated with 20 μl of coated cell suspension as described in the methods section. Non-specific binding (■) was obtained from binding in the presence of benzoquinonium chloride. Specific binding (●) was obtained after subtraction of non-specific from total binding. Specific binding of ^{125}I-αBGT to haemoglobin-coated cells was negligible (not shown). b: Scatchard plot of the data represented in a.

^{125}I-αBGT binding, and cell agglutination was examined using rabbit anti-AChR antiserum. Table I shows that the uptake of AChR increased with increasing amounts of $CrCl_3$ solution used. However, the strongest agglutination was obtained with cells coated using 0.35 ml of $CrCl_3$ solution. Larger volumes of $CrCl_3$ solution yielded cells which agglutinated spontaneously. Therefore, all subsequent coating was carried out with 0.35 ml of $CrCl_3$ solution.

TABLE I

EFFECT OF VOLUME OF $CrCl_3$ SOLUTION USED FOR COUPLING AChR TO SRBC

Vol. of $CrCl_3$ solution used (ml)	Strength of test agglutination [a]	Control [b]	^{125}I-αBGT binding [c] pmol/20 μl of 1% SRBC
0	−	neg	ND [d]
0.1	−	=	0.035
0.2	±	=	0.066
0.25	+	=	0.112
0.30	+ +	=	0.197
0.35	+ + +	=	0.530
0.40	+ +	+	0.722

[a] Haemagglutination performed with rabbit anti-*Torpedo* AChR antiserum.
[b] Haemagglutination performed with normal rabbit serum.
[c] Toxin binding was carried out using excess ^{125}I-αBGT (4.5 pmol).
[d] ND = not detected.

Haemagglutination of AChR-coated red cells

Several polyclonal and monoclonal antisera to AChR were titrated against AChR-coated red cells. The results are summarised in Table II. With polyclonal antisera the agglutination titres correlated well with titres measured by radioimmunoassay. Immunological cross-reactivity between *Torpedo* AChR and receptor from other species was also demonstrable by the agglutination test. Complement-mediated lytic activity was shown by 2 antisera only.

The agglutination characteristics of receptor coated red cells with monoclonal antibodies (mAb) differed from the results obtained with polyclonal antibodies. For example, end-points were achieved with relatively higher amounts of antibodies and there was no correlation between the agglutination and radioimmunoassay titres.

Discussion

The main problem encountered when solubilised membrane proteins are coupled to erythrocytes is the presence of residual detergent which could potentially cause haemolysis. Prior fixation of the cells may overcome this, and recently, Steinitz and Tamir (1985) coated glutaraldehyde-fixed red cells with proteins in the presence of the ionic detergent deoxycholate. However, other workers found that fixed cells could not be coated by the chromic chloride method (Ling et al., 1979). Our results show that, at least in the case of non-ionic detergent-solubilised membrane proteins, dialysis was sufficient to reduce the level of detergent to below that causing cell lysis. Dialysis was preferred to other methods of detergent removal because it does not effect the protein-bound detergent and therefore the protein integrity is preserved. The chromic chloride procedure was successfully used to couple solubilised *Torpedo* AChR to red blood cells. The coated cells behaved well in the agglutination test and

TABLE II

TITRATION OF ANTI-AChR ANTISERA WITH AChR-COATED RED CELLS

Antiserum [a]	Anti-*Torpedo* AChR [b] antibody titre (radioimmunoassay) (nM)	Haemagglutination titre [c]	Complement-*mediated lysis* titre
Normal rabbit serum	ND [d]	1:2	
Rabbit anti-*Torpedo* AChR	14500	1:655360	1:128
Normal sheep serum	ND	–	
Sheep anti-*Torpedo* AChR	6400	1:163840	
Rabbit anti-rat AChR	1517 (42000)	1:40960	
Rabbit anti-foetal calf AChR (1)	17.5 (320)	1:256	1:32
(2)	5.5 (700)	1:8	
Non-immune mouse ascitic fluid	ND	1:2	
Mouse monoclonal anti-*Torpedo* AChR			
A3	2350	1:1024	
B11	170	1:16	
C2	103	1:16	
C7	143	1:1280	

[a] Rabbit anti-rat AChR and foetal calf AChR antisera were raised by immunising animals with receptor purified using the same procedure as for *Torpedo* AChR (methods section).
[b] Values in brackets are antibody titres measured using the primary antigen, i.e., rat AChR and foetal calf AChR.
[c] Titration of anti-*Torpedo* AChR antisera against haemoglobin-coated SRBC showed agglutination in the first well only.
[d] ND = not detected.

were used to detect anti-AChR antibodies with a high level of sensitivity. Correlation between the agglutination test and radioimmunoassay was acceptable in the case of polyclonal antisera. The anomalous results obtained with monoclonal antibodies are not unexpected. In contrast to polyclonal antisera, monoclonal antibody-antigen binding is greatly dependent on the type of assay used. The generally low agglutination titre obtained is most likely due to the fact that the monoclonal antibodies are directed to single antigenic determinants and therefore their cross-linking capability is very much less than that of the polyclonal reagent. The relatively high agglutination titre obtained with mAb-C7 compared with mAb-A3 (see Table II) is a reflection of difference in affinity (another important factor in monoclonal antibody-antigen reactions). Kinetic studies (not presented here) have shown that C7 had a higher binding affinity to *Torpedo* AChR than A3. In general, the agglutina-

tion test offers another method for characterising monoclonal antibodies to AChR in addition to RIA and ELISA.

Red blood cells coated with antigens are useful in the isolation of reactive B-lymphocytes. In preliminary experiments, AChR-coated ox red cells were used in a rosette test to isolate specific B-lymphocytes from (leukophoresed) samples obtained from myasthenic patients whose serum showed relatively high cross-reactivity with *Torpedo* AChR. This selection is important for human monoclonal antibody production by Epstein-Barr virus transformation (Steinitz and Tamir, 1980).

Another practical use of AChR-coated red cells is in the area of ligand-receptor interaction. Ligand interaction with solubilised AChR is usually studied using anion-exchange DE52 cellulose filters, ammonium sulphate precipitation or gel filtration (Schmidt and Raftery, 1973). AChR-SRBC would offer a very convenient and simple procedure for separating bound and free cholinergic ligands.

The main disadvantage with using red cells is their short shelf-life. By handling the cells under aseptic conditions, we have managed to use the cells for up to 4 weeks. Mild glutaraldehyde-fixation of coated cells (Cranage et al., 1983) may permit preservation of the coated cells and increase their shelf-life.

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